



US012383508B2

(12) **United States Patent**
Almarsson et al.

(10) **Patent No.:** US 12,383,508 B2
(45) **Date of Patent:** *Aug. 12, 2025

- (54) **HIGH-PURITY PEG LIPIDS AND USES THEREOF**
- (71) Applicant: **ModernaTX, Inc.**, Cambridge, MA (US)
- (72) Inventors: **Örn Almarsson**, Cambridge, MA (US); **Jin Lim**, Lexington, MA (US); **Eugene Cheung**, Sharon, MA (US); **Jaclyn Milton**, Somerville, MA (US)
- (73) Assignee: **ModernaTX, Inc.**, Cambridge, MA (US)
- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 991 days.
This patent is subject to a terminal disclaimer.
- (21) Appl. No.: **17/277,423**
- (22) PCT Filed: **Sep. 19, 2019**
- (86) PCT No.: **PCT/US2019/051906**
§ 371 (c)(1),
(2) Date: **Mar. 18, 2021**
- (87) PCT Pub. No.: **WO2020/061295**
PCT Pub. Date: **Mar. 26, 2020**

(65) **Prior Publication Data**

US 2022/0031631 A1 Feb. 3, 2022

Related U.S. Application Data

- (60) Provisional application No. 62/745,164, filed on Oct. 12, 2018; provisional application No. 62/733,456, filed on Sep. 19, 2018.
- (51) **Int. Cl.**
A61K 9/51 (2006.01)
A61K 8/86 (2006.01)
C08G 65/332 (2006.01)
- (52) **U.S. Cl.**
CPC *A61K 9/5153* (2013.01); *A61K 8/86* (2013.01); *A61K 9/5123* (2013.01); *C08G 65/3322* (2013.01)
- (58) **Field of Classification Search**
CPC A61K 9/5153; A61K 8/86; A61K 9/5123; A61K 31/7105; A61K 31/7115; A61K 47/14; C08G 65/3322; C08G 65/48; C08L 71/02
- See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

- 5,898,077 A 4/1999 Takahara et al.
6,652,886 B2 11/2003 Ahn et al.
6,696,038 B1 2/2004 Mahala et al.
7,268,120 B1 9/2007 Horton et al.
7,371,404 B2 5/2008 Panzner et al.

7,943,168 B2	5/2011	Schlesinger et al.
8,058,069 B2	11/2011	Yaworski et al.
8,158,601 B2	4/2012	Chen et al.
8,420,123 B2	4/2013	Troiano et al.
8,440,614 B2	5/2013	Castor
8,450,298 B2	5/2013	Mahon et al.
8,460,696 B2	6/2013	Slobodkin et al.
8,460,709 B2	6/2013	Ausborn et al.
8,568,784 B2	10/2013	Lillard et al.
8,569,256 B2	10/2013	Heyes et al.
8,580,297 B2	11/2013	Essler et al.
8,642,076 B2	2/2014	Manoharan et al.
8,652,487 B2	2/2014	Maldonado
8,691,750 B2	4/2014	Constien et al.
8,697,098 B2	4/2014	Perumal et al.
8,709,483 B2	4/2014	Farokhzad et al.
8,710,200 B2	4/2014	Schrum et al.
8,715,736 B2	5/2014	Sachdeva et al.
8,734,832 B2	5/2014	O'Hagan et al.
8,734,846 B2	5/2014	Ali et al.
8,754,062 B2	6/2014	De Fougerolles et al.
8,822,663 B2	9/2014	Schrum et al.
8,980,864 B2	3/2015	Hoge et al.
8,999,380 B2	4/2015	Bancel et al.
9,221,891 B2	12/2015	Bancel et al.
9,283,287 B2	3/2016	Bancel et al.
9,303,079 B2	4/2016	Bancel et al.
9,464,124 B2	11/2016	Bancel et al.
9,512,456 B2	12/2016	Wang et al.
9,533,047 B2	1/2017	de Fougerolles et al.
9,572,896 B2	2/2017	Bancel et al.
9,597,380 B2	3/2017	Chakraborty et al.
9,675,668 B2	6/2017	Bancel et al.
9,803,199 B2	10/2017	Koizumi et al.
9,868,691 B2	1/2018	Benenato et al.
9,872,900 B2	1/2018	Ciararella et al.

(Continued)

FOREIGN PATENT DOCUMENTS

- CN 102068701 A 5/2011
CN 102204920 A 10/2011

(Continued)

OTHER PUBLICATIONS

Lee et al. (Separation and determination of polyethylene glycol fatty acid esters in cosmetics by a reversed-phase HPLC/ELSD, Science Direct, 2008), (Year: 2008).*

(Continued)

Primary Examiner — Robert A Wax

Assistant Examiner — John Seungjai Kwon

(74) *Attorney, Agent, or Firm* — Wolf, Greenfield & Sacks, P.C.

(57) **ABSTRACT**

The present disclosure is based, at least in part, on the discovery that high-purity PEG lipids exhibit superior physical and biological properties, particularly when used in lipid nanoparticle (LNP) formulations. Therefore, the present disclosure provides PEG lipids at a recommended purity, e.g., for use in formulations, such as LNP formulations. The present disclosure also provides LNPs comprising the high-purity PEG lipids, and methods for delivering therapeutic agents to a subject using the same.

(56)

References Cited**U.S. PATENT DOCUMENTS**

10,023,626 B2	7/2018	Bolen et al.	2013/0129785 A1	5/2013	Manoharan et al.
10,064,934 B2	9/2018	Ciaramella et al.	2013/0130348 A1	5/2013	Gu et al.
10,064,935 B2	9/2018	Ciaramella et al.	2013/0142876 A1	6/2013	Howard et al.
10,124,055 B2	11/2018	Ciaramella et al.	2013/0150625 A1	6/2013	Budzik et al.
10,207,010 B2	2/2019	Besin et al.	2013/0156845 A1	6/2013	Manoharan et al.
10,232,055 B2	3/2019	Kariko et al.	2013/0164400 A1	6/2013	Knopov et al.
10,273,269 B2	4/2019	Ciaramella	2013/0171241 A1	7/2013	Geall
10,286,086 B2	5/2019	Roy et al.	2013/0178541 A1	7/2013	Stanton et al.
10,323,076 B2	6/2019	Ellsworth et al.	2013/0183355 A1	7/2013	Jain et al.
10,385,088 B2	8/2019	Fraley et al.	2013/0189351 A1	7/2013	Geall
10,449,244 B2	10/2019	Ciaramella et al.	2013/0195765 A1	8/2013	Gho et al.
10,465,190 B1	11/2019	Chen et al.	2013/0195967 A1	8/2013	Guild et al.
10,493,143 B2	12/2019	Ciaramella et al.	2013/0195968 A1	8/2013	Geall et al.
10,526,629 B2	1/2020	Rabideau et al.	2013/0195969 A1	8/2013	Geall et al.
10,653,712 B2	5/2020	Hoge	2013/0202684 A1	8/2013	Geall et al.
10,653,767 B2	5/2020	Ciaramella et al.	2013/0236533 A1	9/2013	Von Andrian et al.
10,695,419 B2	6/2020	Ciaramella et al.	2013/0236550 A1	9/2013	Ausborn et al.
10,857,105 B2	12/2020	Benenato et al.	2013/0236974 A1	9/2013	De Fougerolles
10,925,958 B2	2/2021	Ciaramella	2013/0243848 A1	9/2013	Lobovkina et al.
11,027,025 B2	6/2021	Hoge et al.	2013/0243867 A1	9/2013	Mahapatra et al.
11,045,540 B2	6/2021	Ciaramella	2013/0245103 A1	9/2013	de Fougerolles et al.
11,103,578 B2	8/2021	Ciaramella et al.	2013/0245104 A1	9/2013	de Fougerolles et al.
11,351,242 B1	6/2022	Lori et al.	2013/0245107 A1	9/2013	de Fougerolles et al.
11,384,352 B2	7/2022	Miracco	2013/0259923 A1	10/2013	Bancel et al.
11,406,703 B2	8/2022	Kramarczyk et al.	2013/0266640 A1	10/2013	De Fougerolles et al.
11,464,848 B2	10/2022	Ciaramella et al.	2013/0274504 A1	10/2013	Colletti et al.
11,485,960 B2	11/2022	Dousis et al.	2013/0302433 A1	10/2013	Bawiec, III et al.
11,497,807 B2	11/2022	Ciaramella et al.	2013/0315831 A1	11/2013	Kallen et al.
11,564,893 B2	1/2023	Smith	2013/0320401 A1	12/2013	Troiano et al.
11,576,961 B2	2/2023	Ciaramella et al.	2013/03295191 A1	11/2013	Troiano et al.
11,643,441 B1	5/2023	Ciaramella et al.	2013/0302432 A1	11/2013	Zale et al.
11,696,946 B2	7/2023	Ciaramella	2013/0302433 A1	11/2013	Troiano et al.
11,752,206 B2	9/2023	Ciaramella et al.	2013/0315831 A1	11/2013	Shi et al.
11,786,607 B2	10/2023	Hoge et al.	2013/0315831 A1	11/2013	Payne et al.
11,851,694 B1	12/2023	Mauger et al.	2013/0336998 A1	12/2013	Kallen et al.
11,866,696 B2	1/2024	Issa et al.	2013/0338210 A1	12/2013	Manoharan et al.
11,872,278 B2	1/2024	Ciaramella et al.	2014/0037714 A1	2/2014	Quay et al.
11,905,525 B2	2/2024	Brito et al.	2014/0039032 A1	2/2014	Kumboyama et al.
11,911,453 B2	2/2024	Ciaramella et al.	2014/0044772 A1	2/2014	MacLachlan et al.
11,912,982 B2	2/2024	Issa et al.	2014/0065204 A1	3/2014	Echeverri et al.
12,070,495 B2	8/2024	Lusso et al.	2014/0065228 A1	3/2014	Hayes et al.
2003/0073619 A1	4/2003	Mahato et al.	2014/0093579 A1	4/2014	Yarowski et al.
2003/0092653 A1	5/2003	Kisich et al.	2014/0113137 A1	4/2014	Zale et al.
2004/0142474 A1	7/2004	Mahala et al.	2014/0121263 A1	5/2014	Podborski et al.
2005/0143336 A1	6/2005	Ramesh et al.	2014/0121393 A1	5/2014	Fitzgerald et al.
2006/0008910 A1	1/2006	MacLachlan et al.	2014/0141070 A1	5/2014	Manoharan et al.
2006/0083780 A1	4/2006	Heyes et al.	2014/0141089 A1	5/2014	Geall et al.
2006/0172003 A1	8/2006	Meers et al.	2014/0141483 A1	5/2014	Bossard et al.
2006/0204566 A1	9/2006	Smyth-Templeton et al.	2014/0142165 A1	5/2014	Grayson et al.
2007/0252295 A1	11/2007	Panzner et al.	2014/0142254 A1	5/2014	Fonnun et al.
2009/0042825 A1	2/2009	Matar et al.	2014/0147432 A1	5/2014	Bancel et al.
2009/0042829 A1	2/2009	Matar et al.	2014/0148502 A1	5/2014	Bancel et al.
2010/0130588 A1	5/2010	Yaworski et al.	2014/0193482 A1	7/2014	Bancel et al.
2011/0200582 A1	8/2011	Baryza et al.	2014/0206752 A1	7/2014	Afeyan et al.
2011/0244026 A1	10/2011	Guild et al.	2014/0206753 A1	7/2014	Guild et al.
2011/0256175 A1	10/2011	Hope et al.	2014/0255472 A1	9/2014	Geall et al.
2012/0021042 A1	1/2012	Panzner et al.	2014/0308304 A1	10/2014	Manoharan et al.
2012/0065252 A1	3/2012	Schrum et al.	2014/0378538 A1	12/2014	Bancel
2012/0101148 A1	4/2012	Aking et al.	2015/0051268 A1	2/2015	Bancel et al.
2012/0157500 A1	6/2012	Weikang	2015/0056253 A1	2/2015	Bancel et al.
2012/0172411 A1	7/2012	Heyes et al.	2015/0141499 A1	5/2015	Bancel et al.
2012/0177724 A1	7/2012	Irvine et al.	2015/0307542 A1	10/2015	Roy et al.
2012/0178702 A1	7/2012	Huang	2015/0315541 A1	11/2015	Bancel et al.
2012/0251618 A1	10/2012	Schrum et al.	2015/0376115 A1	12/2015	Ansell et al.
2012/0295832 A1	11/2012	Constien et al.	2016/0022580 A1	1/2016	Ramsay et al.
2013/0017223 A1	1/2013	Hope et al.	2016/0024140 A1	1/2016	Issa et al.
2013/0064894 A1	3/2013	Martin et al.	2016/0024141 A1	1/2016	Shahrokh et al.
2013/0065942 A1	3/2013	Matar et al.	2016/0032273 A1	2/2016	Hoge et al.
2013/0090372 A1	4/2013	Budzik et al.	2016/0038612 A1	2/2016	Geall et al.
2013/0102034 A1	4/2013	Schrum et al.	2016/0194368 A1	7/2016	Hoge et al.
2013/0115273 A1	5/2013	Yang et al.	2016/0194625 A1	7/2016	Hoge et al.
2013/0115274 A1	5/2013	Knopov et al.	2016/0243221 A1	8/2016	Hoge et al.
2013/0116307 A1	5/2013	Heyes et al.	2016/0271272 A1	9/2016	Bancel et al.
2013/0122104 A1	5/2013	Yaworski et al.	2016/0317647 A1	11/2016	Ciaramella et al.
2013/0123338 A1	5/2013	Heyes et al.	2016/0331828 A1	11/2016	Ciaramella et al.

(56)

References Cited

U.S. PATENT DOCUMENTS

2017/0043037 A1	2/2017	Kariko et al.	2021/0268086 A1	9/2021	Zhong et al.
2017/0065675 A1	3/2017	Bancel et al.	2021/0309976 A1	10/2021	Dousis et al.
2017/0130255 A1	5/2017	Wang et al.	2021/0378980 A1	12/2021	Horphota et al.
2017/0202979 A1	7/2017	Chakraborty et al.	2022/0031631 A1	2/2022	Almarsson et al.
2017/0204152 A1	7/2017	Nelson et al.	2022/0047518 A1	2/2022	Hennessy et al.
2017/0340724 A1	11/2017	Ciaramella et al.	2022/0054653 A1	2/2022	Martini et al.
2018/0000953 A1	1/2018	Almarsson et al.	2022/0062175 A1	3/2022	Smith et al.
2018/0002393 A1	1/2018	Bancel et al.	2022/0125899 A1	4/2022	Ashburn et al.
2018/0021258 A1	1/2018	Graham et al.	2022/0145381 A1	5/2022	Elich et al.
2018/0028664 A1	2/2018	Besin et al.	2022/0236253 A1	7/2022	Hopson
2018/0237849 A1	8/2018	Thompson	2022/0241399 A1	8/2022	Lusso et al.
2018/0243225 A1	8/2018	Ciaramella	2022/0347292 A1	11/2022	Panther et al.
2018/0243230 A1	8/2018	Smith	2022/0348900 A1	11/2022	Shamashkin et al.
2018/0256628 A1	9/2018	Hoge et al.	2022/0349006 A1	11/2022	Amato et al.
2018/0271795 A1	9/2018	Martini et al.	2023/0000970 A1	1/2023	Nachbagauer et al.
2018/0271970 A1	9/2018	Ciaramella et al.	2023/0142529 A1	5/2023	White et al.
2018/0273977 A1	9/2018	Mousavi et al.	2023/0181481 A1	6/2023	White et al.
2018/0274009 A1	9/2018	Marquardt et al.	2023/0190761 A1	6/2023	Brader et al.
2018/0280496 A1	10/2018	Ciaramella et al.	2023/0212645 A1	7/2023	Marquardt et al.
2018/0289792 A1	10/2018	Ciaramella et al.	2023/0287437 A1	9/2023	Smith et al.
2018/0303929 A1	10/2018	Ciaramella et al.	2023/0338506 A1	10/2023	Shaw et al.
2018/0311336 A1	11/2018	Ciaramella et al.	2023/0346914 A1	11/2023	Stewart-Jones et al.
2018/0311343 A1	11/2018	Huang et al.	2023/0355743 A1	11/2023	Stewart-Jones et al.
2018/0312549 A1	11/2018	Ciaramella	2024/0100145 A1	3/2024	Bollman et al.
2018/0318409 A1	11/2018	Valiante et al.	2024/0100151 A1	3/2024	Carfi et al.
2018/0363019 A1	12/2018	Hoge	2024/0139309 A1	5/2024	Carfi et al.
2018/0369374 A1	12/2018	Frederick et al.	2024/0173400 A1	5/2024	Ciaramella et al.
2018/0371047 A1	12/2018	Ticho et al.	2024/0181030 A1	6/2024	Himansu et al.
2019/0002890 A1	1/2019	Martini et al.	2024/0207392 A1	6/2024	Chandramouli et al.
2019/0008938 A1	1/2019	Ciaramella et al.	2024/0209068 A1	6/2024	Deal et al.
2019/0085368 A1	3/2019	Bancel et al.	2024/0226028 A1	7/2024	Goldman et al.
2019/0099481 A1	4/2019	Ciaramella et al.	2024/0226277 A1	7/2024	Nachbagauer et al.
2019/0125839 A1	5/2019	Frederick et al.	2024/0229109 A1	7/2024	Rabideau et al.
2019/0175517 A1	6/2019	Martini et al.	2024/0238211 A1	7/2024	Brader et al.
2019/0175727 A1	6/2019	Huang et al.	2024/0263226 A1	8/2024	Schmitt
2019/0192646 A1	6/2019	Cohen et al.	2024/0285754 A1	8/2024	Stewart-Jones
2019/0192653 A1	6/2019	Hoge et al.	2024/0293534 A1	9/2024	Stewart-Jones
2019/0275170 A1	9/2019	Benenato et al.	2024/0299531 A1	9/2024	Stewart-Jones
2019/0298657 A1	10/2019	Martini et al.	2024/0358819 A1	10/2024	Stewart-Jones
2019/0298658 A1	10/2019	Benenato			
2019/0300906 A1	10/2019	Martini et al.			
2019/0314292 A1	10/2019	Benenato et al.			
2019/0314493 A1	10/2019	Ciaramella et al.			
2019/0336452 A1	11/2019	Brader			
2019/0336595 A1	11/2019	Ciaramella			
2019/0351040 A1	11/2019	Valiante et al.			
2019/0382774 A1	12/2019	Hoge et al.			
2019/0390181 A1	12/2019	Benenato et al.			
2020/0030432 A1	1/2020	Ciaramella et al.			
2020/0032274 A1	1/2020	Mauger et al.			
2020/0038499 A1	2/2020	Narayanan et al.			
2020/0054737 A1	2/2020	Ciaramella et al.			
2020/0069599 A1	3/2020	Smith et al.			
2020/0069793 A1	3/2020	Ciaramella			
2020/0069794 A1	3/2020	Ciaramella et al.			
2020/0071689 A1	3/2020	Miracco			
2020/0085916 A1	3/2020	Martini et al.			
2020/0109420 A1	4/2020	Brito et al.			
2020/0129445 A1*	4/2020	Patel	C12N 15/113		
2020/0129608 A1	4/2020	Ciaramella et al.			
2020/0129615 A1	4/2020	Ciaramella et al.			
2020/0163878 A1	5/2020	Baumhof et al.			
2020/0239869 A1	7/2020	Issa et al.			
2020/0254086 A1	8/2020	Hoge et al.			
2020/0282047 A1	9/2020	Ciaramella et al.			
2020/0306191 A1	10/2020	Schariter et al.			
2020/0338004 A1	10/2020	Hansson et al.			
2020/0368162 A1	11/2020	Martini			
2021/0046173 A1	2/2021	Ciaramella et al.			
2021/0087135 A1	3/2021	Benenato et al.			
2021/0163919 A1	6/2021	Issa et al.			
2021/0187097 A1	6/2021	Ciaramella et al.			
2021/0206818 A1	7/2021	Huang et al.			
2021/0217484 A1	7/2021	Giessel et al.			
2021/0228707 A1	7/2021	Mektar et al.			

FOREIGN PATENT DOCUMENTS

CN	102813929 A	12/2012
CN	104644555 A	5/2015
EP	1404860 A2	4/2004
EP	2073848 A2	7/2009
JP	H06-287137 A	10/1994
JP	2002-069821 A	3/2002
WO	WO 1999/014346 A2	3/1999
WO	WO 1999/052503 A2	10/1999
WO	WO 2005/034979 A2	4/2005
WO	WO 2008/042973 A2	4/2008
WO	WO 2009/024599 A1	2/2009
WO	WO 2009/127060 A1	10/2009
WO	WO 2010/042877 A1	4/2010
WO	WO 2010/054406 A1	5/2010
WO	WO 2010/088537 A2	8/2010
WO	WO 2010/129709 A1	11/2010
WO	WO 2010/144710 A1	12/2010
WO	WO 2010/141069 A2	* 12/2010 A61K 31/77
WO	WO 2011/017108 A2	2/2011
WO	WO 2011/068810 A1	6/2011
WO	WO 2011/127255 A1	10/2011
WO	WO 2011/140627 A1	11/2011
WO	WO 2012/006376 A2	1/2012
WO	WO 2012/006378 A1	1/2012
WO	WO 2012/030901 A1	3/2012
WO	WO 2012/031043 A1	3/2012
WO	WO 2012/031046 A2	3/2012
WO	WO 2012/099755 A1	7/2012
WO	WO 2012/129483 A1	9/2012
WO	WO 2012/149252 A2	11/2012
WO	WO 2012/149393 A2	11/2012
WO	WO 2012/153338 A2	11/2012
WO	WO 2012/170889 A1	12/2012
WO	WO 2012/170930 A1	12/2012
WO	WO 2013/006825 A1	1/2013
WO	WO 2013/006834 A1	1/2013
WO	WO 2013/006837 A1	1/2013

(56)	References Cited						
FOREIGN PATENT DOCUMENTS							
WO	WO 2013/006838 A1	1/2013	WO	WO 2017/070616 A2	4/2017		
WO	WO 2013/006842 A2	1/2013	WO	WO 2017/070618 A1	4/2017		
WO	WO 2013/033438 A2	3/2013	WO	WO 2017/070620 A2	4/2017		
WO	WO 2013/033563 A1	3/2013	WO	WO 2017/070622 A1	4/2017		
WO	WO 2013/049328 A1	4/2013	WO	WO 2017/070623 A1	4/2017		
WO	WO 2013/052167 A2	4/2013	WO	WO 2017/070624 A1	4/2017		
WO	WO 2013/056132 A2	4/2013	WO	WO 2017/070626 A2	4/2017		
WO	WO 2013/057715 A1	4/2013	WO	WO-2017075531 A1 *	5/2017 A61K 31/713	
WO	WO 2013/059496 A1	4/2013	WO	WO 2017/112865 A1	6/2017		
WO	WO 2013/059922 A1	5/2013	WO	WO-2017099823 A1 *	6/2017 A61K 31/715	
WO	WO 2013/064911 A2	5/2013	WO	WO 2017/127750 A1	7/2017		
WO	WO 2013/066903 A1	5/2013	WO	WO 2017/201333 A1	11/2017		
WO	WO 2013/067537 A1	5/2013	WO	WO 2018/053209 A1	3/2018		
WO	WO 2013/086322 A1	6/2013	WO	WO 2018/075980 A1	4/2018		
WO	WO 2013/086354 A1	6/2013	WO	WO 2018/081459 A1	5/2018		
WO	WO 2013/086373 A1	6/2013	WO	WO 2018/081462 A1	5/2018		
WO	WO 2013/086526 A1	6/2013	WO	WO 2018/089801 A1	5/2018		
WO	WO 2013/087083 A1	6/2013	WO	WO 2018/089851 A2	5/2018		
WO	WO 2013/090186 A1	6/2013	WO	WO 2018/107088 A2	6/2018		
WO	WO 2013/090648 A1	6/2013	WO	WO 2018/111967 A1	6/2018		
WO	WO 2013/093648 A2	6/2013	WO	WO 2018/144082 A1	8/2018		
WO	WO 2013/135359 A1	9/2013	WO	WO 2018/144778 A1	8/2018		
WO	WO 2013/143555 A1	10/2013	WO	WO 2018/151816 A1	8/2018		
WO	WO 2013/143683 A1	10/2013	WO	WO 2018/157009 A1	8/2018		
WO	WO 2013/148186 A1	10/2013	WO	WO 2018/170245 A1	9/2018		
WO	WO 2013/148541 A1	10/2013	WO	WO 2018/170256 A1	9/2018		
WO	WO 2013/149141 A1	10/2013	WO	WO 2018/170260 A1	9/2018		
WO	WO 2013/151669 A1	10/2013	WO	WO 2018/170270 A1	9/2018		
WO	WO 2013/151672 A2	10/2013	WO	WO 2018/170347 A1	9/2018		
WO	WO 2013/158127 A1	10/2013	WO	WO 2018/175783 A1	9/2018		
WO	WO 2013/158579 A1	10/2013	WO	WO 2018/187590 A2	10/2018		
WO	WO 2013/166498 A1	11/2013	WO	WO 2018/200737 A1	11/2018		
WO	WO 2013/173693 A1	11/2013	WO	WO 2018/232355 A1	12/2018		
WO	WO 2013/185069 A1	12/2013	WO	WO 2018/232357 A1	12/2018		
WO	WO 2014/008334 A1	1/2014	WO	WO 2019/018765 A1	1/2019		
WO	WO 2014/026284 A1	2/2014	WO	WO 2019/036670 A1	2/2019		
WO	WO 2014/028487 A1	2/2014	WO	WO 2019/036682 A1	2/2019		
WO	WO 2014/054026 A1	4/2014	WO	WO 2019/036683 A1	2/2019		
WO	WO 2014/071072 A2	5/2014	WO	WO 2019/036685 A1	2/2019		
WO	WO 2014/072997 A1	5/2014	WO	WO 2019/103993 A1	5/2019		
WO	WO 2014/081507 A1	5/2014	WO	WO 2019/148101 A1	8/2019		
WO	WO 2014/144196 A1	9/2014	WO	WO 2020/006242 A1	1/2020		
WO	WO 2014/144711 A1	9/2014	WO	WO 2020/056370 A1	3/2020		
WO	WO 2014/152027 A1	9/2014	WO	WO 2020/061284 A1	3/2020		
WO	WO 2014/152030 A1	9/2014	WO	WO 2020/061295 A1	3/2020		
WO	WO 2014/152774 A1	9/2014	WO	WO 2020/061367 A1	3/2020		
WO	WO 2014/159813 A1	10/2014	WO	WO 2020/097291 A1	5/2020		
WO	WO 2014/160243 A1	10/2014	WO	WO 2020/172239 A1	8/2020		
WO	WO 2014/172045 A1	10/2014	WO	WO 2020/185811 A1	9/2020		
WO	WO 2014/182661 A2	11/2014	WO	WO 2020/190750 A1	9/2020		
WO	WO 2014/210356 A1	12/2014	WO	WO 2020/243561 A1	12/2020		
WO	WO 2015/085318 A2	6/2015	WO	WO 2021/030533 A1	2/2021		
WO	WO 2015/095340 A1	6/2015	WO	WO 2021/050864 A1	3/2021		
WO	WO 2015/095346 A1	6/2015	WO	WO 2021/055811 A1	3/2021		
WO	WO 2015/130584 A2	9/2015	WO	WO 2021/055849 A1	3/2021		
WO	WO 2015/148247 A1	10/2015	WO	WO 2021/155243 A1	8/2021		
WO	WO 2015/164674 A1	10/2015	WO	WO 2021/155274 A1	8/2021		
WO	WO 2015/164786 A1	10/2015	WO	WO 2021/159040 A2	8/2021		
WO	WO 2016/037053 A1	3/2016	WO	WO 2021/159130 A2	8/2021		
WO	WO 2016/118724 A1	7/2016	WO	WO 2021/211343 A1	10/2021		
WO	WO 2016/118725 A1	7/2016	WO	WO 2021/222304 A1	11/2021		
WO	WO 2016/164762 A1	10/2016	WO	WO 2021/231929 A1	11/2021		
WO	WO 2016/201377 A1	12/2016	WO	WO 2021/231963 A1	11/2021		
WO	WO 2017/011773 A2	1/2017	WO	WO 2021/237084 A1	11/2021		
WO	WO 2017/015457 A1	1/2017	WO	WO 2021/247817 A1	12/2021		
WO	WO 2017/015463 A2	1/2017	WO	WO 2022/032154 A2	2/2022		
WO	WO 2017/019935 A1	2/2017	WO	WO 2022/197624 A1	9/2022		
WO	WO 2017/020026 A1	2/2017	WO	WO 2022/212442 A1	10/2022		
WO	WO 2017/031232 A1	2/2017	WO	WO 2022/212711 A2	10/2022		
WO	WO 2017/031241 A1	2/2017	WO	WO 2022/221335 A1	10/2022		
WO	WO 2017/049245 A2	3/2017	WO	WO 2022/221336 A1	10/2022		
WO	WO 2017/062513 A1	4/2017	WO	WO 2022/221440 A1	10/2022		
WO	WO 2017/066789 A1	4/2017	WO	WO 2022/226277 A1	10/2022		
WO	WO 2017/070601 A1	4/2017	WO	WO 2022/226318 A1	10/2022		
WO	WO 2017/070613 A1	4/2017	WO	WO 2022/232585 A1	11/2022		

(56)

References Cited

FOREIGN PATENT DOCUMENTS

WO WO 2022/241103 A1 11/2022
 WO WO 2022/245888 A1 11/2022
 WO WO 2022/266010 A1 12/2022
 WO WO 2022/266012 A1 12/2022
 WO WO 2022/266389 A1 12/2022
 WO WO 2023/283642 A2 1/2023
 WO WO 2023/283645 A1 1/2023
 WO WO 2023/283651 A1 1/2023
 WO WO 2023/014649 A1 2/2023
 WO WO 2023/018773 A1 2/2023
 WO WO 2023/018923 A1 2/2023
 WO WO 2023/019181 A1 2/2023
 WO WO 2023/056401 A1 4/2023
 WO WO 2023/069625 A1 4/2023
 WO WO 2023/069895 A1 4/2023
 WO WO 2023/069900 A1 4/2023
 WO WO 2023/076658 A1 5/2023
 WO WO 2023/081311 A1 5/2023
 WO WO 2023/092069 A1 5/2023
 WO WO 2023/107999 A2 6/2023
 WO WO 2023/114307 A1 6/2023
 WO WO 2023/132885 A1 7/2023
 WO WO 2023/137149 A1 7/2023
 WO WO 2023/150256 A1 8/2023
 WO WO 2023/154818 A1 8/2023
 WO WO 2023/196914 A1 10/2023
 WO WO 2023/201204 A1 10/2023
 WO WO 2023/201294 A1 10/2023
 WO WO 2023/201296 A1 10/2023
 WO WO 2023/212696 A1 11/2023
 WO WO 2023/225524 A1 11/2023
 WO WO 2023/250119 A1 12/2023
 WO WO 2024/010993 A1 1/2024
 WO WO 2024/015890 A1 1/2024
 WO WO 2024/026005 A1 2/2024
 WO WO 2024/030369 A1 2/2024
 WO WO 2024/050483 A1 3/2024
 WO WO 2024/097874 A1 5/2024
 WO WO 2024/123978 A1 6/2024
 WO WO 2024/151811 A1 7/2024
 WO WO 2024/163465 A1 8/2024
 WO WO 2024/191860 A2 9/2024
 WO WO 2024/206835 A1 10/2024
 WO WO 2024/215721 A1 10/2024

OTHER PUBLICATIONS

- Fang et al. (Development of Lipid-Shell and Polymer Core Nanoparticles with Water-Soluble Salidroside for Anti-Cancer Therapy. *Int. J. Mol. Sci.* 2014). (Year: 2014).*
- Sabnis et al. (A Novel Amino Lipid Series for mRNA Delivery: Improved Endosomal Escape and Sustained Pharmacology and Safety in Non-human Primates, *Molecular Therapy*, Jun. 2018 (Year: 2018).*
- Delmas et al. (Preparation and characterization of highly stable lipid nanoparticles with amorphous core of tuneable viscosity, *Journal of Colloid and Interface Science*, 2011 (Year: 2011).*
- International Search Report and Written Opinion for Application No. PCT/US2019/051906 dated Feb. 19, 2020.
- Abu Lila et al., Application of polyglycerol coating to plasmid DNA lipoplex for the evasion of the accelerated blood clearance phenomenon in nucleic acid delivery. *J Pharm Sci.* Feb. 2014;103(2):557-66. doi: 10.1002/jps.23823. Epub Dec. 17, 2013.
- Abu Lila et al., Use of polyglycerol (PG), instead of polyethylene glycol (PEG), prevents induction of the accelerated blood clearance phenomenon against long-circulating liposomes upon repeated administration. *Int J Pharm.* Nov. 1, 2013;456(1):235-42. doi: 10.1016/j.ijpharm.2013.07.059. Epub Aug. 5, 2013.
- Akinc et al., Targeted Delivery of RNAi Therapeutics With Endogenous and Exogenous Ligand-Based Mechanisms, *Mol Ther.* 2009 17:872-879.
- Anderson, D.M. et al., Stability of mRNA/cationic lipid lipoplexes in human and rat cerebrospinal fluid: methods and evidence for nonviral mRNA gene delivery to the central nervous system. *Hum Gene Ther.* Feb. 10, 2003;14(3):191-202.
- Andreakos et al., Amphoteric liposomes enable systemic antigen-presenting cell-directed delivery of CD40 antisense and are therapeutically effective in experimental arthritis. *Arthritis Rheum.* Apr. 2009;60(4):994-1005. doi: 10.1002/art.24434.
- Andries et al., Comparison of the gene transfer efficiency of mRNA/GL67 and pDNA/GL67 complexes in respiratory cells. *Mal Pharmaceutics.* 2012; 9: 2136-2145.
- Bag, Recovery of normal protein synthesis in heat-shocked chicken myotubes by liposome-mediated transfer of mRNAs. *Can. J. Biochem. Cell Biol.* 1985; 63(3): 231-235.
- Belliveau, N.M., et al., Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. *Mol Ther Nucleic Acids.* Aug. 2012; 1(8): e37.
- Bettinger, T. et al., Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. *Nucleic Acids Res.* Sep. 15, 2001;29(18):3882-91.
- Bogers et al., Potent immune responses in rhesus macaques induced by nonviral delivery of a self-amplifying RNA vaccine expressing HIV type 1 envelope with a cationic nanoemulsion. *J Infect Dis.* Mar. 15, 2015;211(6):947-55. doi:10.1093/infdis/jiu522. Epub Sep. 18, 2014.
- Bolhassani et al. , Improvement of Different Vaccine Delivery Systems for Cancer Therapy, *Molecular Cancer*, Biomed Central, London, GB, 2011, vol. 10, No. 3, pp. 1-20.
- Chamberlain et al., Recurrent lymphomatous meningitis treated with intra-CSF rituximab and liposomal ara-C. *J Neurooncol.* Feb. 2009;91(3):271-7. doi: 10.1007/s11060-008-9707-1. Epub Sep. 27, 2008.
- Chen, D., et al., Rapid discovery of potent siRNA-containing lipid nanoparticles enabled by controlled microfluidic formulation. *J Am Chem Soc.* 2012; 134: 6948-6951.
- Corazzelli et al., Biweekly rituximab, cyclophosphamide, vincristine, non-pegylated liposome-encapsulated doxorubicin and prednisone (R-COMP-14) in elderly patients with poor-risk diffuse large B-cell lymphoma and moderate to high 'life threat' impact cardiopathy. *Br J Haematol.* Sep. 2011;154(5):579-89. doi: 10.1111/j.1365-2141.2011.08786.x. Epub Jun. 28, 2011.
- Cross, Without these lipid shells, there would be no mRNA vaccines for COVID-19. *Chem Eng News.* Mar. 6, 2021; 99(8). 4 pages.
- Cun et al., Preparation and characterization of poly(DL-lactide-co-glycolide) nanoparticles for siRNA delivery. *International Journal of Pharmaceutics* 390 (2010) 70-75.
- Dahlman et al., In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight, *Nature Nanotechnology*, 2014, No. vol. #, pp. 1-8.
- Delehaney, Peptides for Specific Intracellular Delivery and Targeting of Nanoparticles: Implications for Developing Nanoparticle-Mediated Drug Delivery, *Future Science, Therapeutic Delivery*, 2010, vol. 1, No. 3, pp. 411-433.
- Delmas et al., Encapsulation and Release Behavior from Lipid Nanoparticles: Model Study with Nile Red Fluorophore. *J. Colloid Sci. Biotechnol.* 2012;1:16-25.
- Felgner, Cationic lipid/polynucleotide condensates for in vitro and in vivo polynucleotide delivery—the cytoseptins. *J. of Liposome Research.* 1993; 3(1): 3-16.
- Felgner, et al., Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U SA.* Nov. 1987;84(21):7413-7.
- Felgner, Particulate systems and polymers for in vitro and in vivo delivery of polynucleotides. *Adv. Drug Delivery Rev.* 1990; 5(3): 163-187.
- Fleeton et al., Self-replicative RNA vaccines elicit protection against influenza A virus, respiratory syncytial virus, and a tickborne encephalitis virus. *J Infect Dis.* May 1, 2001;183(9):1395-8. Epub Mar. 30, 2001.
- Geall et al., Nonviral delivery of self-amplifying RNA vaccines. *Proc Natl Acad Sci U S A.* Sep. 4, 2012;109(36):14604-9. doi: 10.1073/pnas.1209367109. Epub Aug. 20, 2012.

(56)

References Cited**OTHER PUBLICATIONS**

- Gindy et al., Stabilization of Ostwald ripening in low molecular weight amino lipid nanoparticles for systemic delivery of siRNA therapeutics. *Mol Pharm.* Nov. 3, 2014;11(11):4143-53. doi: 10.1021/mp500367k. Epub Oct. 15, 2014.
- Gluzman et al., Esterification of stearic acid with polyethylene glycols. *Zhurnal Prikladnoi Khimii, Maik Nauka: Rossiiskaya Akademiya Nauk.* Jan. 1, 1968;41(1):167-170.
- Hashimoto et al., Relationship between the concentration of anti-polyethylene glycol (PEG) immunoglobulin M (IgM) and the intensity of the accelerated blood clearance (ABC) phenomenon against PEGylated liposomes in mice. *Biol Pharm Bull.* 2015;38(3):417-24. doi: 10.1248/bpb.b14-00653.
- He et al., Synthesis and Separation of Diastereomers of Ribonucleoside 5'-(alpha-P-Borano)triposphates. *J Org Chem.* Aug. 21, 1998;63(17):5769-5773.
- Hecker et al., Non-Viral DNA and mRNA Gene Delivery to the CNS Pre-Operatively for Neuroprotection and Following Neurotrauma. *Molecular Therapy.* 2004; 9, S258-S258.
- Hussein et al., Synthesis, Quantum Chemical Calculations and Properties of Nonionic and Nonionic-Anionic Surfactants Based on Fatty Alkyl Succinate. *Journal of Surfactants and Detergents* vol. 17, pp. 615-627(2014).
- Juliano et al., Cell-targeting and cell-penetrating peptides for delivery of therapeutic and imaging agents. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology.* May/Jun. 2009; 1(3): 324-335.
- Kallen et al., A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. *Ther Adv Vaccines.* Jan. 2014;2(1): 10-31. doi: 10.1177/2051013613508729.
- Kallen et al., A novel, disruptive vaccination technology: self-adjuvanted RNAActive(RTM.) vaccines. *Hum Vaccin Immunother.* Oct. 2013;9(10):2263-76. doi: 10.4161/hv.25181. Epub Jun. 4, 2013. Review.
- Kanapathipillai et al., Nanoparticle targeting of anti-cancer drugs that alter intracellular signaling or influence the tumor microenvironment, *Adv. Drug Deliv. Rev.* (2014), , pp. 1-12.
- Kariko et al., Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res.* vol. 39, No. 21, Nov. 1, 2011, pp. e142-1, XP002696190.
- Kariko et al., In vivo protein expression from mRNA delivered into adult rat brain. *J. of Neuroscience Methods.* Jan. 2001; 105(1): 77-86.
- Kariko et al., Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability, *Molecular Therapy, Nature Publishing Group, GB*, vol. 16, No. 11, Nov. 1, 2008 (Nov. 1, 2008), pp. 1833-1840.
- Kariko et al., Phosphate-enhanced transfection of cationic lipid-complexed mRNA and plasmid DNA. *Biochim Biophys Acta.* Mar. 2, 1998; 1369(2):320-34.
- Kauffman et al., Efficacy and immunogenicity of unmodified and pseudouridine-modified mRNA delivered systemically with lipid nanoparticles in vivo. *Biomaterials.* Dec. 2016;109:78-87. doi: 10.1016/j.biomaterials.2016.09.006. Epub Sep. 25, 2016.
- Keown et al., Methods for Introducing DNA into Mammalian Cells. *Methods in Enzymology.* 1990, 185:527-37.
- Kirpotin et al., Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res.* 2006; 66: 6732-6740.
- Kowalski et al., Delivering the Messenger: Advances in Technologies for Therapeutic mRNA Delivery. *Molecular Therapy* vol. 27 No. 4 Apr. 2019.
- Kozlinski et al., Bioreducible Cationic Polymer-Based Nanoparticles for Efficient and Environmentally Triggered Cytoplasmic siRNA Delivery to Primary Human Brain Cancer Cells, *ACS Nano*, 2014, vol. 8, 'No. 4',pp. 3232-3241.
- Krause et al., Prevention of the hemodynamic effects of iopromide-carrying liposomes in rats and pigs. *Invest Radiol.* Aug. 2000;35(8):493-503.
- Kulkarni et al., Lipid Nanoparticles Enabling Gene Therapies: From Concepts to Clinical Utility. *Nucleic Acid Ther.* Jun. 2018;28(3):146-157. doi: 10.1089/nat.2018.0721. Epub Apr. 23, 2018.
- Lai et al., Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev.* Feb. 27, 2009; 61(2): 158-171.
- Lai et al., Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *PNAS.* Jan. 30, 2007; 104(5): 1482-1487.
- Lee et al., Lipid Nanoparticle siRNA Systems for Silencing the Androgen Receptor in Human Prostate Cancer in Vivo, *International Journal of Cancer.* 2012, vol. 131, pp. 781-790.
- Lee et al., Separation and determination of polyethylene glycol fatty acid esters in cosmetics by a reversed-phase HPLC/ELSD. *Talanta.* Feb. 15, 2008;74(5):1615-20. doi: 10.1016/j.talanta.2007.10.020. Epub Oct. 18, 2007.
- Lehto et al., Cell-penetrating peptides for the delivery of nucleic acids. *Expert Opin. Drug Deliv.* Jul. 2012; 9(7): 823-836.
- Lewis, Dynamic Polyconjugates (DPC) Technology: An elegant solution to the siRNA delivery problem. Arrowhead Research Corp (NASDAQ: ARWR). Nov. 2011.
- Li et al., Overcoming obstacles to develop effective and safe siRNA therapeutics. *Expert Opin Biol Ther.* May 2009; 9(5): 609-19.
- Li et al., Preparation and gene delivery of alkaline amino acids-based cationic liposomes. *Arch Pharm Res.* Jul. 2008;31(7):924-31. Epub Aug. 14, 2008.
- Lian et al., Trends and developments in liposome drug delivery systems. *J Pharm Sci.* Jun. 2001;90(6):667-80.
- Lopez-Berestein et al., Treatment of systemic fungal infections with liposomal amphotericin B. *Arch Intern Med.* Nov. 1989; 149(11):2533-6.
- MacLachlan, Lipid Nanoparticle-mediated delivery of messenger RNA. Presentation. 1st International mRNA Health Conference. Tubingen, Germany. Oct. 24, 2013. http://files.shareholder.com/downloads/ABEA-50QJTB/2628241206x0x699789/47-543d12-db34-4e6e-88a9-f3ae5d97b1d2/MacLachlan_mRNA_Conf_2013.pdf. Last accessed Dec. 22, 2016.
- Madden et al., Systemic delivery of mRNA therapeutics using lipid nanoparticles (LNP): improved potency for novel LNP and influence of route of administration on protein expression. 2nd International mRNA Health Conference. Nov. 12, 2014.<https://acuitastx.com/wp-content/uploads/2015/01/Poster-Second-International-mRNA-Health-Conference.pdf>. 1 page.
- Magee et al., Marked stimulation of lymphocyte-mediated attack on tumor cells by target-directed liposomes containing immune RNA, *Cancer Res.* 1978, 38(4):1173-6.
- Malone et al., Cationic liposome-mediated RNA transfection. *Proc Natl Acad Sci U SA.* Aug. 1989;86 (16):6077-81.
- Marina et al., Dose escalation and pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in children with solid tumors: a pediatric oncology group study. *Clin Cancer Res.* Feb. 2002;8(2):413-8.
- Martinon et al., Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. *Eur J Immunol.* Jul. 1993;23(7):1719-22.
- Maurer et al., Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. *Biophys J.* May 2001; 80(5): 2310-2326.
- Michel T. et al.: "Cationic Nanoliposomes Meet mRNA: Efficient Delivery of Modified mRNA Using Hemocompatible and Stable Vectors for Therapeutic Applications" *Molecular Therapy Nucleic Acids,* 2017, vol. 8, pp. 459-468, <http://dx.doi.org/10.1016/j.omtn.2017.07.013>.
- Midoux et al., Lipid-based mRNA vaccine delivery systems. *Expert Rev Vaccines.* Feb. 2015;14(2):221-34. doi: 10.1586/14760584.2015.986104. Epub Dec. 26, 2014. Review.
- Mishra et al., Improved leishmanicidal effect of phosphorothioate antisense oligonucleotides by LDL-mediated delivery. *Biochim Biophys Acta.* Nov. 7, 1995;1264(2):229-37.

(56)

References Cited**OTHER PUBLICATIONS**

- Mockey et al., mRNA-based cancer vaccine: prevention of B16 melanoma progression and metastasis by systemic injection of MART1 mRNA histidylated lipopolplexes, *Cancer Gene Therapy*, 2007, 14, pp. 802-814.
- Nair et al., Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro, *J Exp Med*. Feb. 1, 1992;175(2):609-12.
- Oberli et al., Lipid Nanoparticle Assisted mRNA Delivery for Potent Cancer Immunotherapy. *Nano Lett*. Mar. 8, 2017;17(3):1326-1335. doi: 10.1021/acs.nanolett.6b03329. Epub Dec. 5, 2016.
- Oja et al., Doxorubicin entrapped within liposome-associated antigens results in a selective inhibition of the antibody response to the linked antigen. *Biochim Biophys Acta*. Sep. 29, 2000;1468(1-2):31-40.
- Oster et al., Comparative study of DNA encapsulation into PLGA microparticles using modified double emulsion methods and spray drying techniques. *Journal of Microencapsulation*, May 2005; 22(3): 235-244.
- Parker et al., Targeting of Polyelectrolyte RNA Complexes to Cell Surface Integrins as an Efficient, Cytoplasmic Transfection Mechanism, *Journal of Bioactive and Compatible Polymers*, Jul. 2002, pp. 1-10.
- Pollard et al., Type I IFN counteracts the induction of antigen-specific immune responses by lipid-based delivery of mRNA vaccines. *Mol Ther*. Jan. 2013; 21 (1): 251-259.
- Popov et al., Multivalent rituximab lipid nanoparticles as improved lymphoma therapies: indirect mechanisms of action and in vivo activity. *nNanomedicine (Lond)*. Nov. 2011;6(9):1575-91. doi: 10.2217/nmm.11.50 Epub Oct. 20, 2011.
- Pulford et al., Liposome-siRNA-peptide complexes cross the blood-brain barrier and significantly decrease PrP^C on neuronal cells and PrP^{RES} in infected cell cultures. *PLoS One*. 2010; 5(6): e11085.
- Reichmuth et al., mRNA Vaccine Delivery Using Lipid Nanoparticles. *Ther Deliv*. 2016;7(5):319-34. doi: 10.4155/tde-2016-0006.
- Riccardi et al., "Dressing up" an Old Drug: An Aminoacyl Lipid for the Functionalization of Ru(III)-Based Anticancer Agents. *ACS Biomater. Sci. Eng.* 2018, 4, 1, 163-174.
- Saito et al., Distribution of liposomes into brain and rat brain tumor models by convection-enhanced delivery monitored with magnetic resonance imaging. *Cancer Res*. Apr. 2004; 64: 2572-2579.
- Sakuma et al., Mucoadhesion of polystyrene nanoparticles having surface hydrophilic polymeric chains in the gastrointestinal tract. *Int J Pharm*. Jan. 25, 1999;177(2):161-72.
- Schott et al., Viral and non-viral approaches for transient delivery of mRNA and proteins. *Current Gene Ther*. 2011; 11 (5): 382-398.
- Shea et al., Synthesis, hybridization properties and antiviral activity of lipid-oligodeoxynucleotide conjugates. *Nucleic Acids Res*. Jul. 11, 1990;18(13):3777-83.
- Shimizu et al., Transport of PEGylated liposomes from the splenic marginal zone to the follicle in the induction phase of the accelerated blood clearance phenomenon. *Immunobiology*. May 2013;218(5):725-32. doi: 10.1016/j.imbio.2012.08.274. Epub Aug. 23, 2012.
- Shin et al., Recent Advances in RNA Therapeutics and RNA Delivery Systems Based on Nanoparticles. *Adv. Therap.*, Nov. 2018;1(7):1800065. Review.
- Strobel et al., Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes. *Gene Ther*. Dec. 2000; 7(23): 2028-2035.
- Taguchi et al., Effect of Repeated Injections of Adenosine Diphosphate-Encapsulated Liposomes Coated with a Fibrinogen .gamma.-Chain Dodecapeptide Developed as a Synthetic Platelet Substitute on Accelerated Blood Clearance in a Healthy and an Anticancer Drug-Induced Thrombocytopenia Rat Model. *J Pharm Sci*. Sep. 2015;104(9):3084-91. doi: 10.1002/jps.24418. Epub Mar. 9, 2015.
- Taguchi et al., Hemoglobin vesicles, polyethylene glycol (PEG)ylated liposomes developed as a red blood cell substitute, do not induce the accelerated blood clearance phenomenon in mice. *Drug Metab Dispos*. Nov. 2009;37(11):2197-203. doi: 10.1124/dmd.109.028852. Epub Aug. 13, 2009.
- Tavernier et al., mRNA as gene therapeutic: How to control protein expression. *J. of Controlled Release*. Mar. 2011; 150(3): 238-247.
- Terada et al., Characterization of Lipid Nanoparticles Containing Ionizable Cationic Lipids Using Design-of-Experiments Approach. *Langmuir*. Jan. 26, 2021;37(3):1120-1128. doi: 10.1021/acs.langmuir.0c03039. Epub Jan. 13, 2021.
- Thess et al., Sequence-engineered mRNA Without Chemical Nucleoside Modifications Enables an Effective Protein Therapy in Large Animals. *Mol Ther*. Sep. 2015;23(9):1456-64. doi: 10.1038/mt.2015.103. Epub Jun. 8, 2015.
- Torchilin et al., Multifunctional and Stimuli-Sensitive Pharmaceutical Nanocarriers, *Eur J. Pharm Biopharm*, 2009, vol. 71, No. 3, pp. 431-444.
- Torrecilla et al., Lipid Nanoparticles as Carriers for RNAi Against Viral Infections: Current Status and Future Perspectives. *Biomed Res Int*. 2014;2014:161794. doi: 10.1155/2014/161794. Epub Aug. 12, 2014.
- Tracy, "Progress in the Development of LNP Delivery for siRNA Advancing LNPs to the Clinic," International Liposome Research Days Meeting, Vancouver, Canada. Aug. 2010, pp. 1-52.
- Treat et al., Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, 1989. 353-65.
- Uzgun et al., PEGylation improves nanoparticle formation and transfection efficiency of messenger RNA. *Pharm Res*. Sep. 2011; 28(9): 2223-2232.
- Viklund et al., Enzymatic synthesis of surfactants based on polyethylene glycol and stearic or 12-hydroxystearic acid. *Journal of Molecular Catalysis B Enzymatic* 27(2):51-53 . Feb. 2004.
- Wan et al., Lipid nanoparticle delivery systems for siRNA-based therapeutics. *Drug Deliv Transl Res*. Feb. 2014;4(1):74-83. doi: 10.1007/s13346-013-0161-z.
- Wang et al., Chapter 3: Lipid Nanoparticles for the Delivery of Nucleic Acids. Book: *Nanoparticulate Drug Delivery Systems: Strategies, Technologies, and Applications*. 2013. 29 pages.
- Wang et al., Enhanced bioavailability and efficiency of curcumin for the treatment of asthma by its formulation in solid lipid nanoparticles. *Int J Nanomedicine*. 2012;7:3667-77. doi: 10.2147/IJN.S30428. Epub Jul. 17, 2012.
- Wang et al., Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy. *Mol Ther*. Feb. 2013;21(2):358-67. doi: 10.1038/mt.2012.250. Epub Dec. 11, 2012.
- Weilhammer et al., The use of nanolipoprotein particles to enhance the immunostimulatory properties of innate immune agonists against lethal influenza challenge. *Biomaterials*. Dec. 2013;34(38):10305-18. doi: 10.1016/j.biomaterials.2013.09.038. EpubSep. 27, 2013.
- Wilson et al., Real time measurement of PEG shedding from lipid nanoparticles in serum via NMR spectroscopy. *Mol Pharm*. Feb. 2, 2015;12(2):386-92. doi: 10.1021/mp500400k. Epub Jan. 12, 2015.
- Yamamoto et al., Current prospects for mRNA gene delivery, *European Journal of Pharmaceutics and Biopharmaceutics* 71 (2009) 484-489.
- Zeng et al., Formulation and Delivery Technologies for mRNA Vaccines. *Curr Top Microbiol Immunol*. Jun. 2, 2020;10.1007/82_2020_217. doi: 10.1007/82_2020_217. 37 pages.
- Zhao et al., Chapter Two: Lipid Nanoparticles for Gene Delivery. Book: *Advances in Genetics*. Elsevier, 2014. 24 pages.
- Zhigaltsev et al., Bottom-Up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing. *Langmuir*. Feb. 21, 2012; 28(7): 3633-3640.
- Zimmermann et al., Electrolyte- and pH-stabilities of aqueous solid lipid nanoparticle (SL.N.TM.) dispersions in artificial gastrointestinal media. *Eur J Pharm Biopharm*. Sep. 2001;52(2):203-10.
- Zohra et al., Drastic effect of nanoapatite particles on liposome-mediated mRNA delivery to mammalian cells. *Analytical Biochem*. Oct. 2005; 345(1): 164-166.

(56)

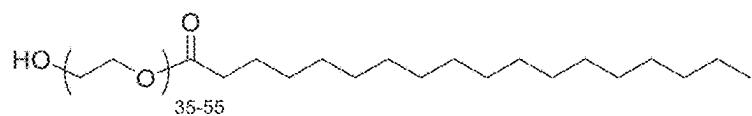
References Cited

OTHER PUBLICATIONS

- Zohra et al., Effective delivery with enhanced translational activity synergistically accelerates mRNA-based transfection. Biochem Biophys Res Comm. Jun. 2007; 358(1): 373-378.
- [No Author Listed], Polyethylene Glycol Monostearate. Japanese Pharmaceutical Excipients. Mar. 29, 2018. p. 772.
- Sabnis et al., A Novel Amino Lipid Series for mRNA Delivery: Improved Endosomal Escape and Sustained Pharmacology and Safety in Non-human Primates. Mol Ther. Jun. 6, 2018;26(6):1509-1519. doi: 10.1016/j.ymthe.2018.03.010. Epub Mar. 14, 2018.
- U.S. Appl. No. 17/583,674, filed Jan. 25, 2022, Besin et al.
- U.S. Appl. No. 17/277,452, filed Mar. 18, 2021, Hennessy et al.
- U.S. Appl. No. 18/556,221, filed Oct. 19, 2023, Brader et al.
- U.S. Appl. No. 18/288,328, filed Oct. 25, 2023, Goldman et al.
- U.S. Appl. No. 17/726,971, filed Apr. 22, 2022, Hennessy.
- U.S. Appl. No. 18/682,685, filed Feb. 9, 2024, Smith.
- U.S. Appl. No. 18/719,476, filed Jun. 13, 2024, Brader.

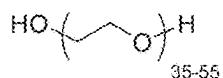
* cited by examiner

PEG Lipids of Formula (I):

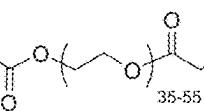
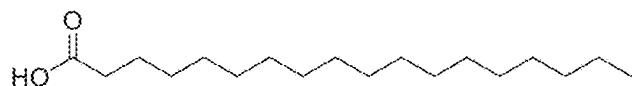


(I), HO-PEG-Stearate

Potential Associated Impurities:



Free PEG



Stearate-PEG-Stearate

Figure 1

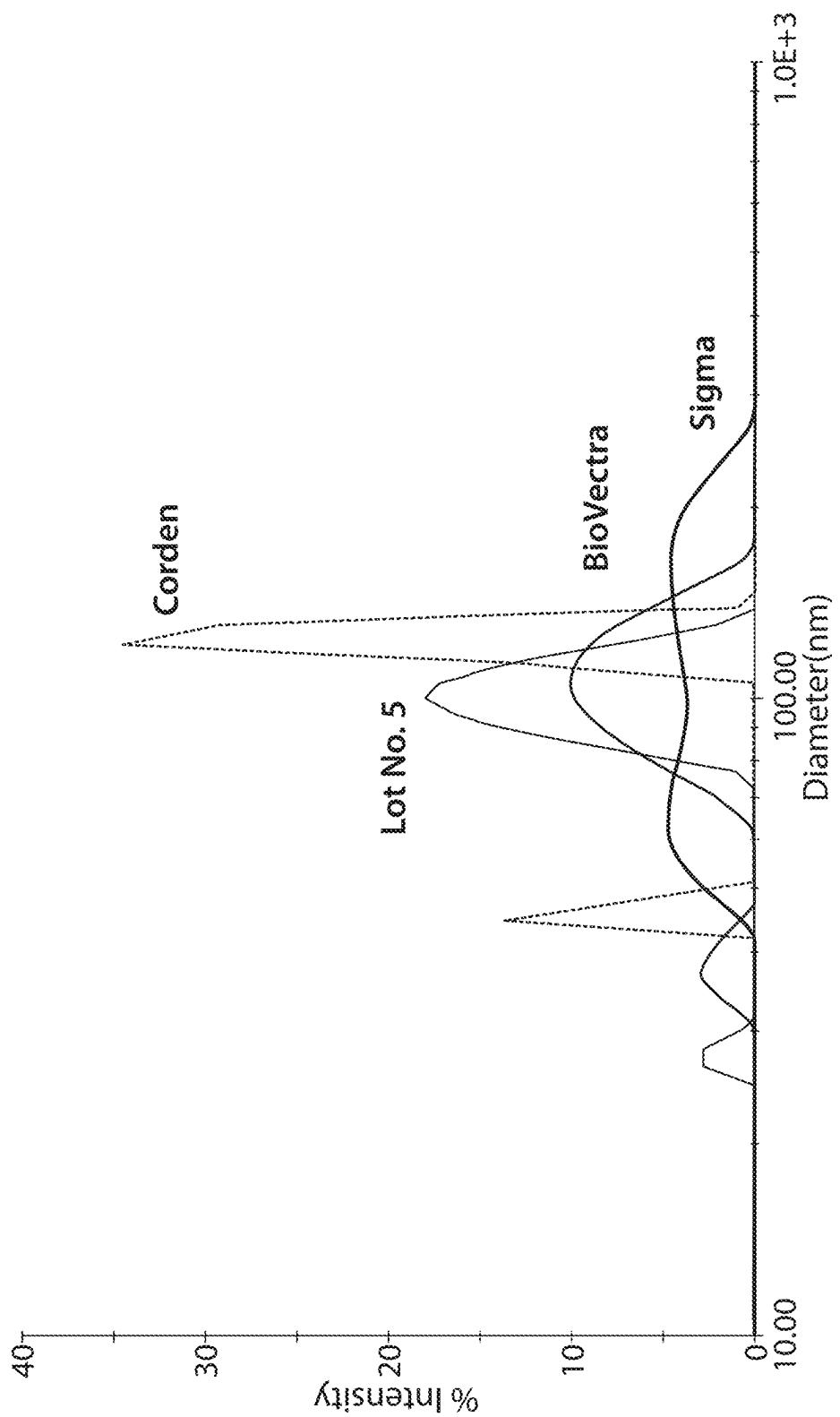


Figure 2

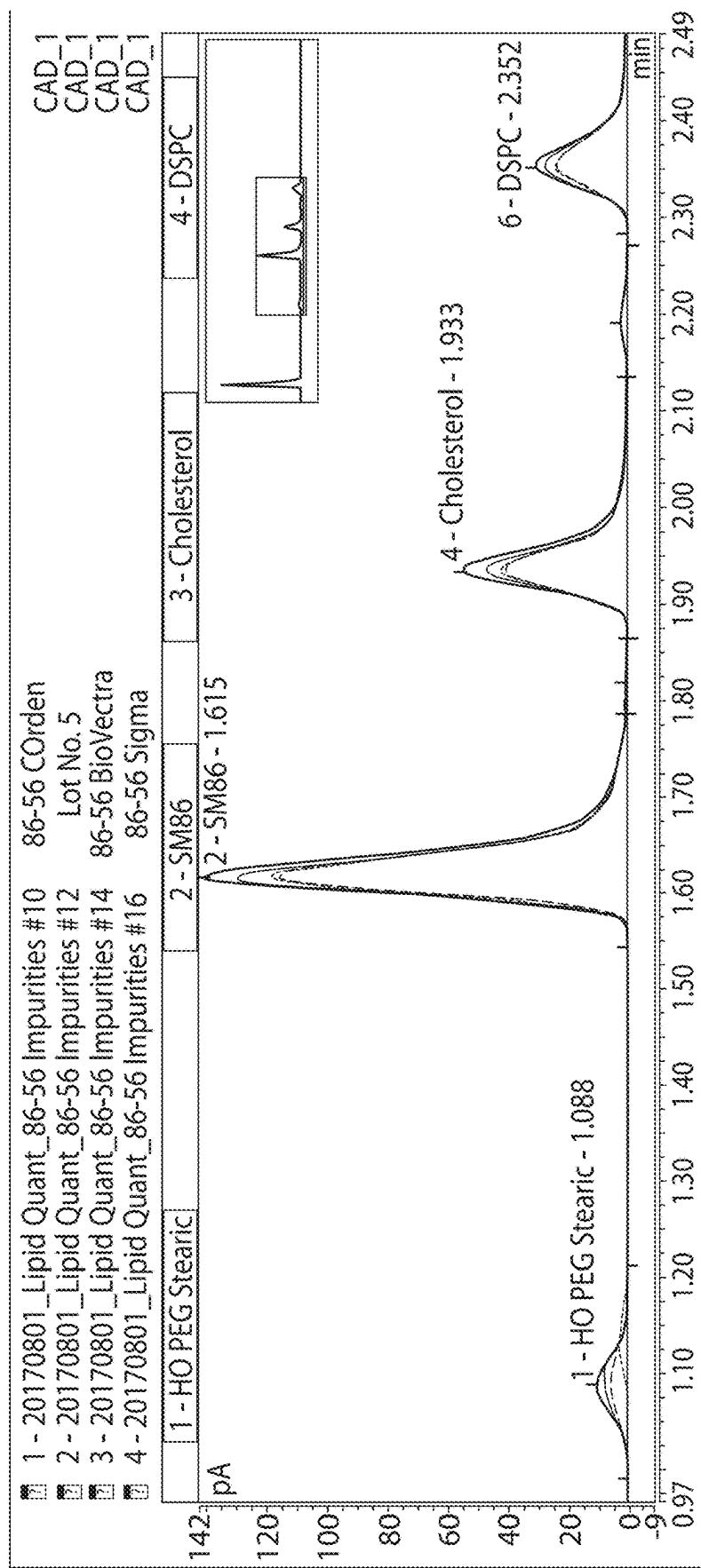


Figure 3

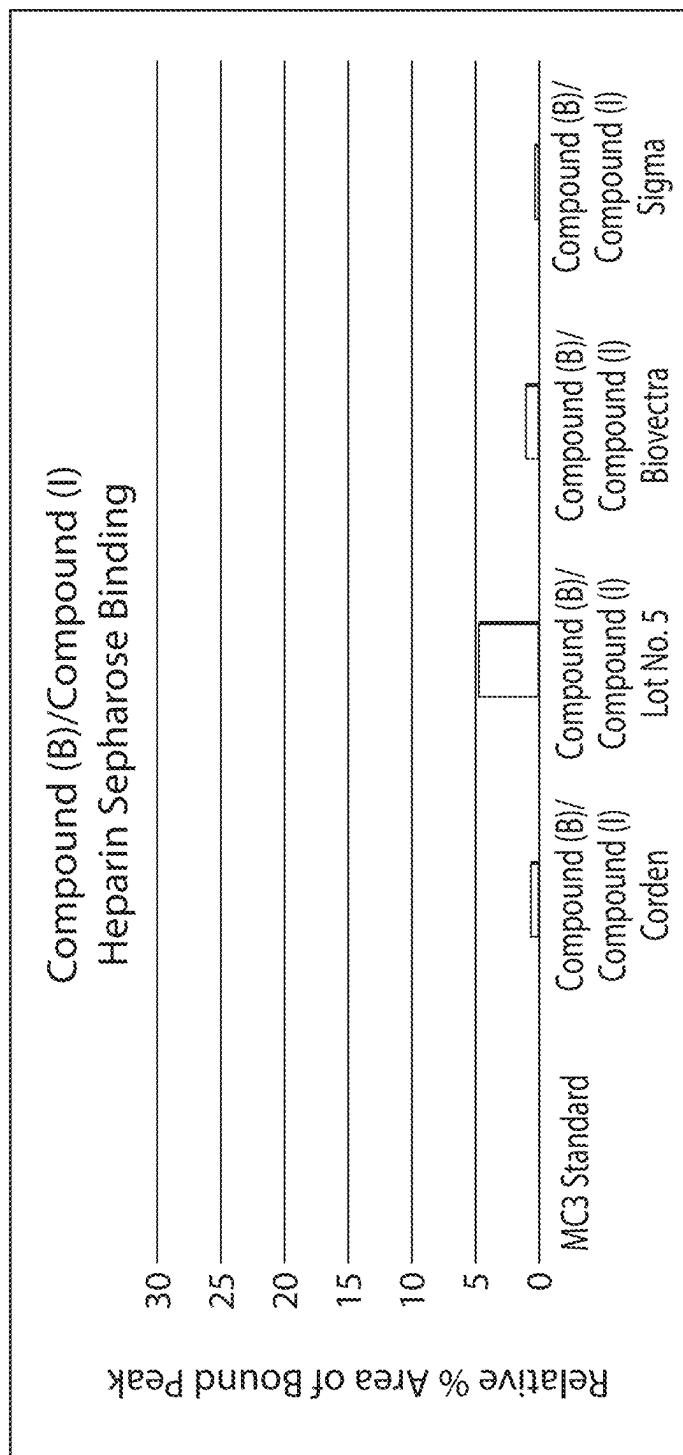


Figure 4

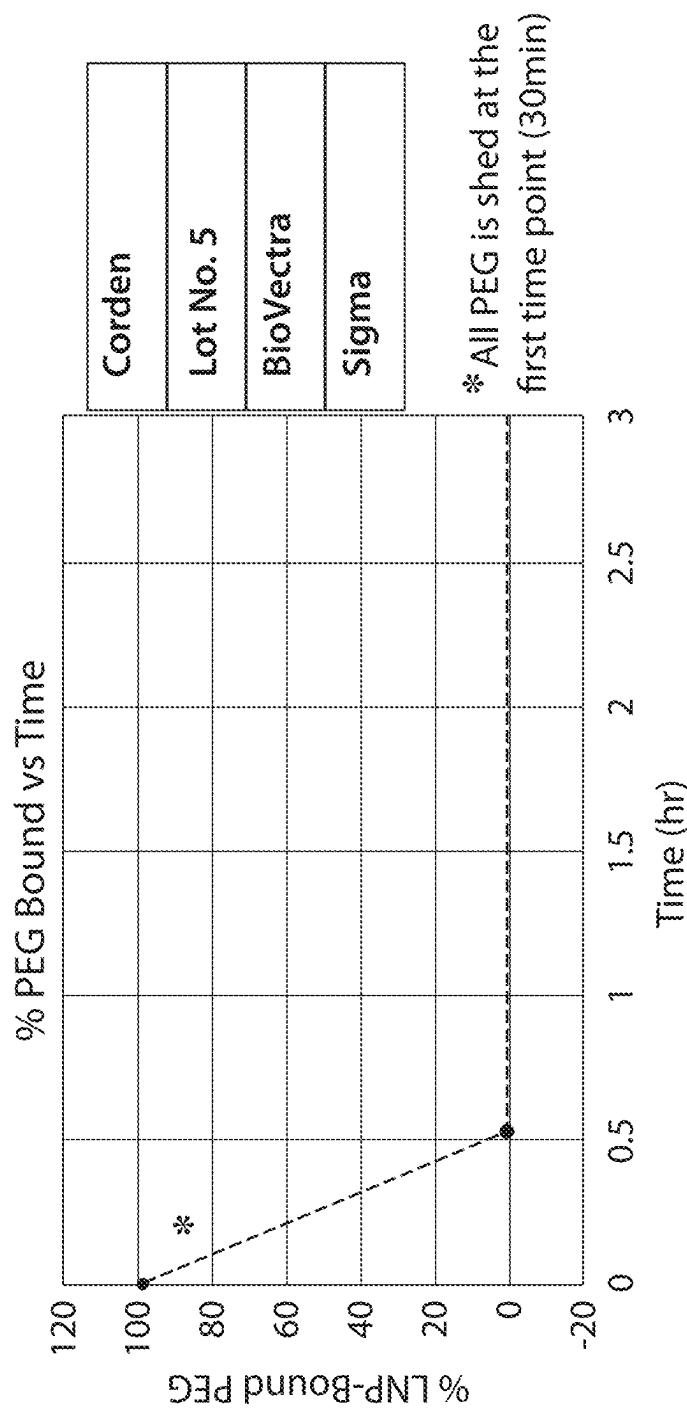


Figure 5

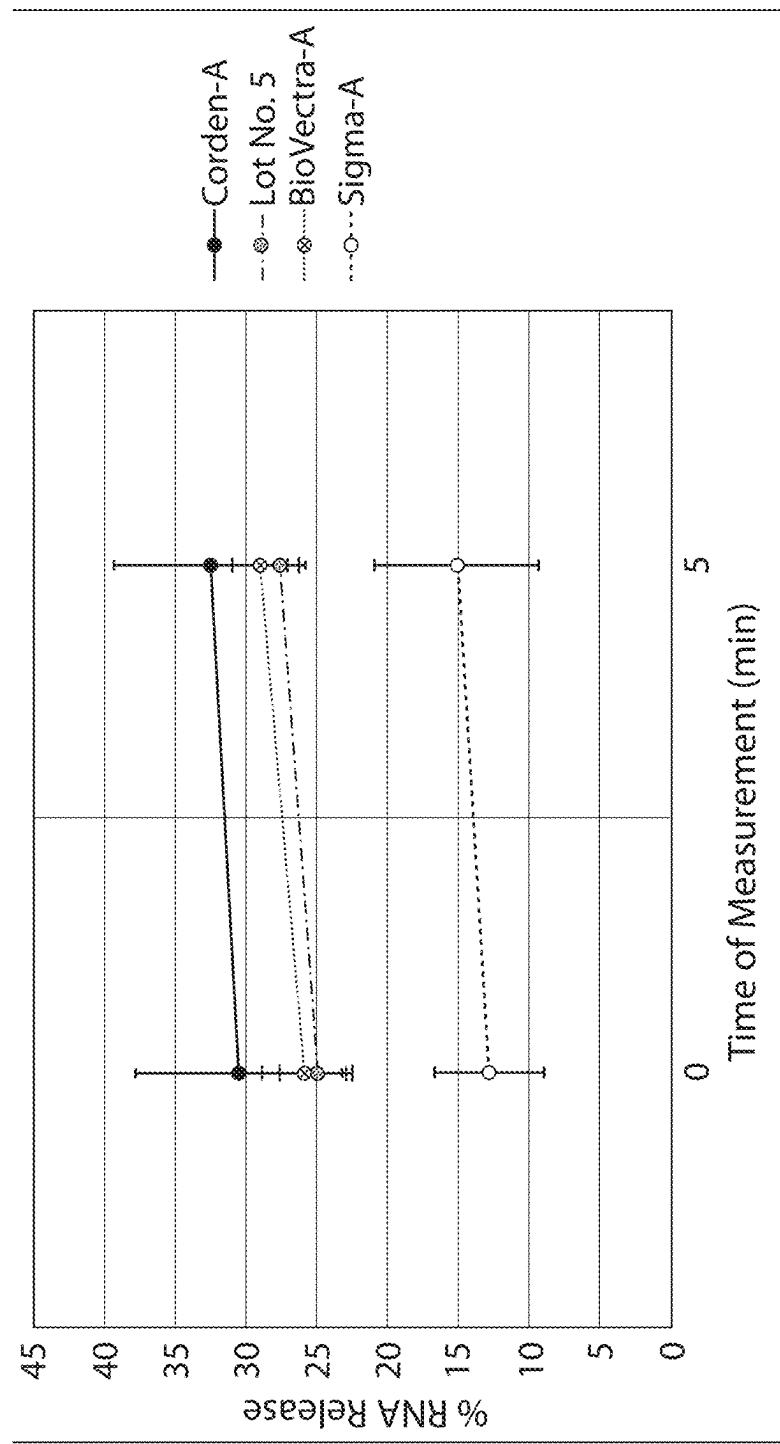


Figure 6

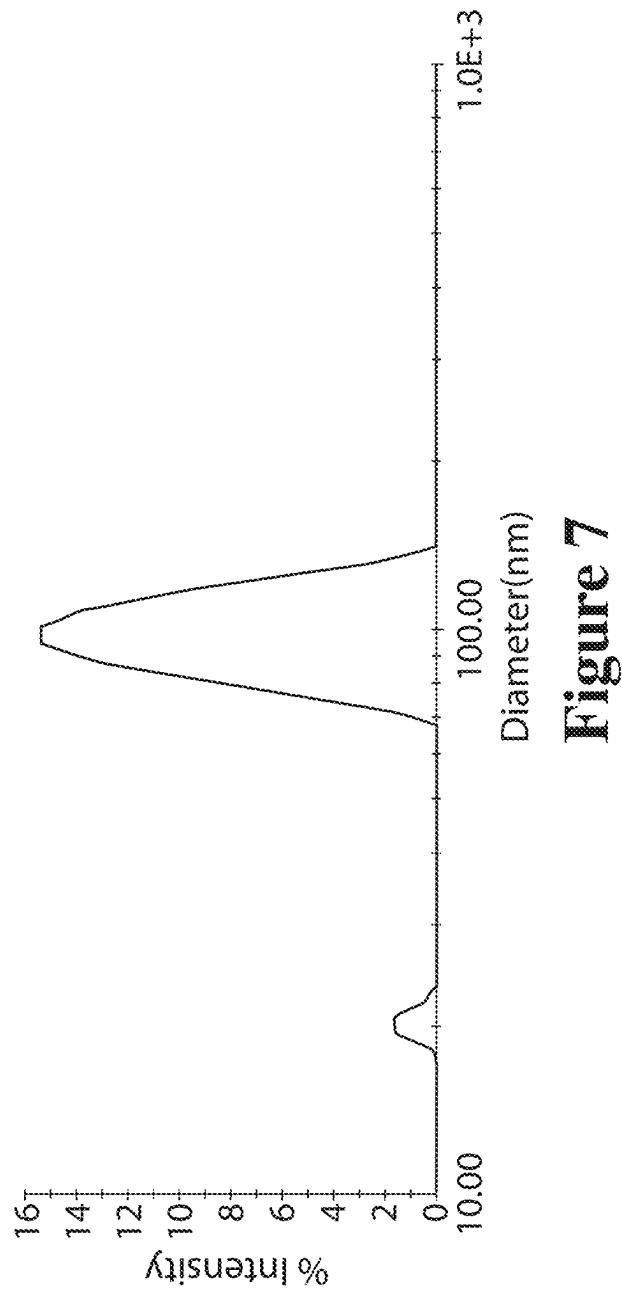


Figure 7

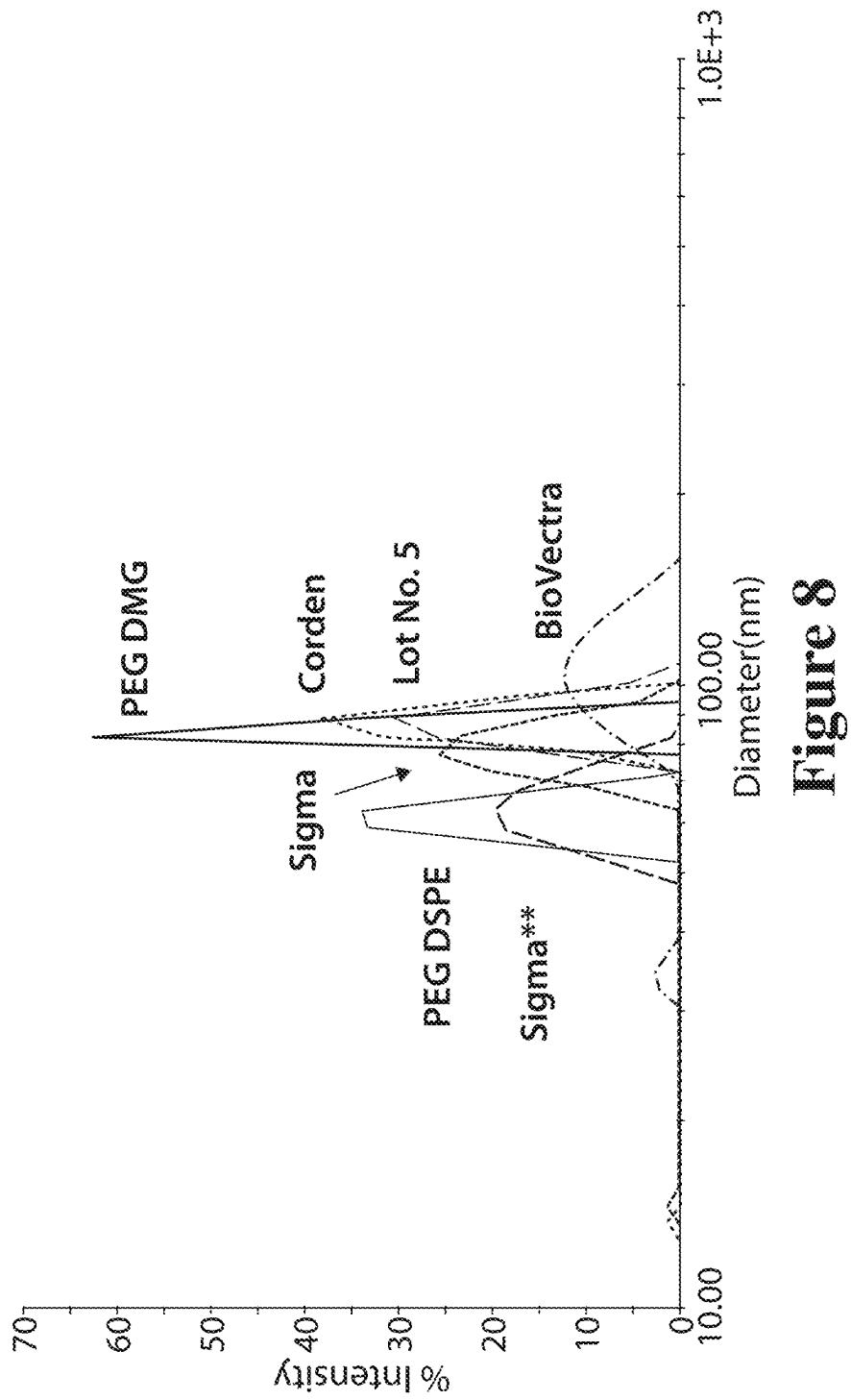
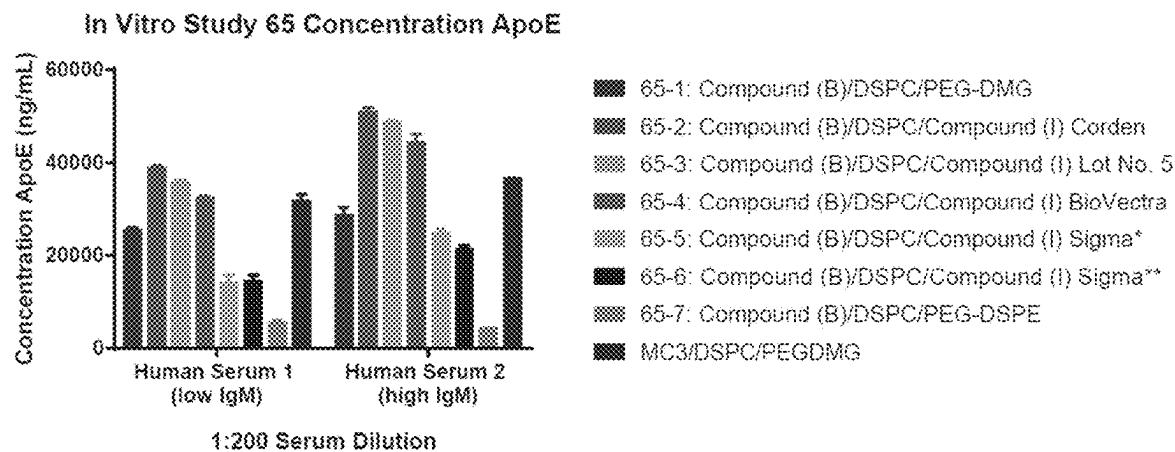
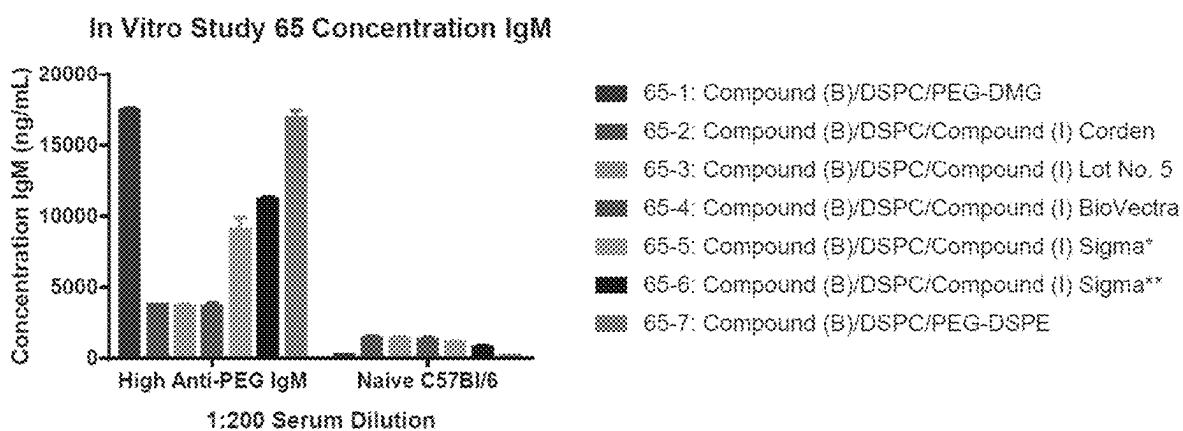


Figure 8

**Figure 9****Figure 10**

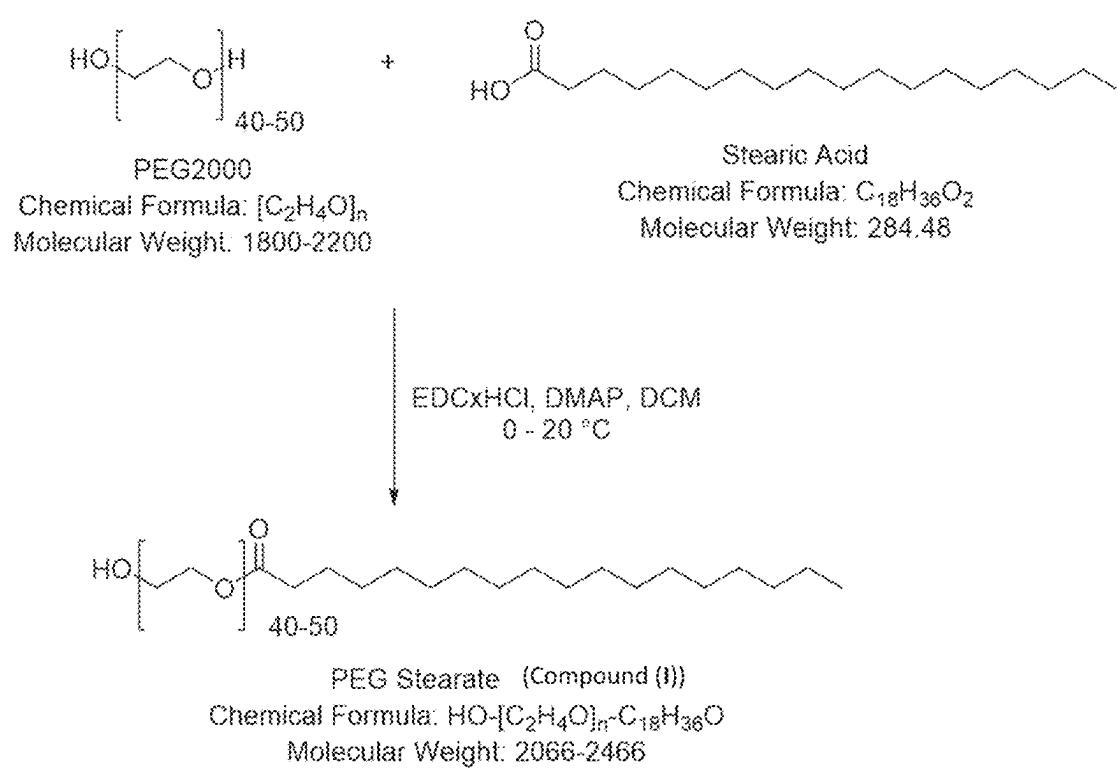


Figure 11

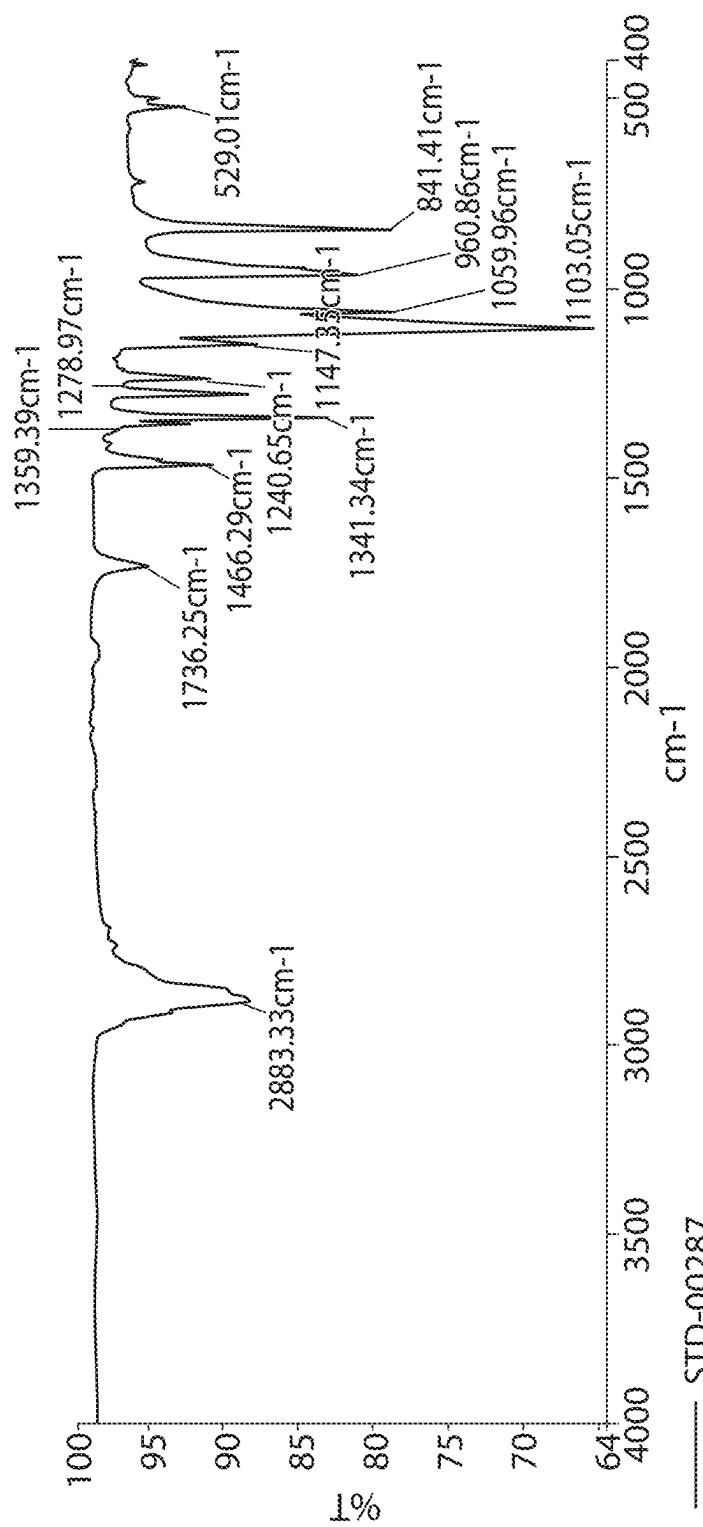


Figure 12A

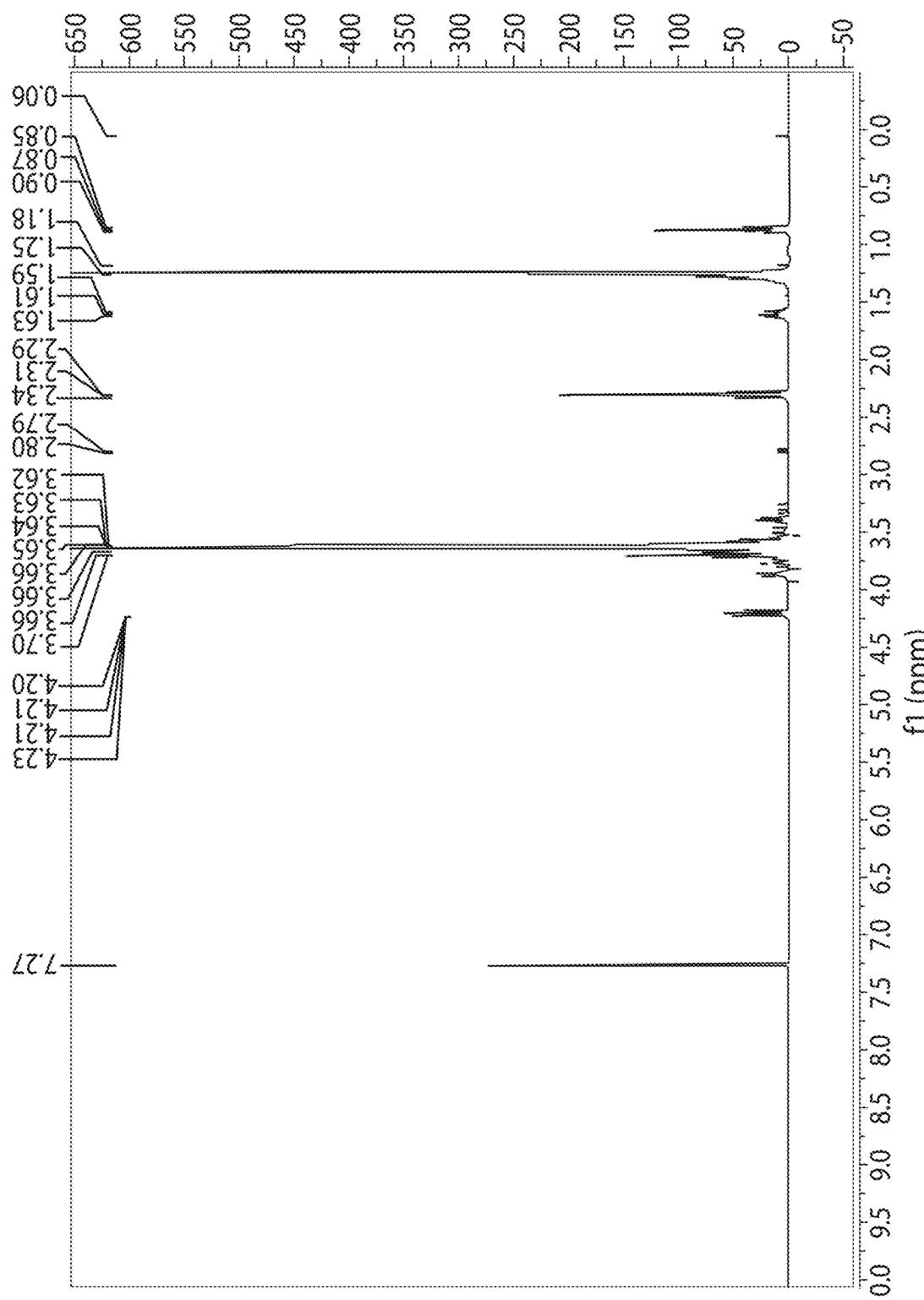


Figure 12B

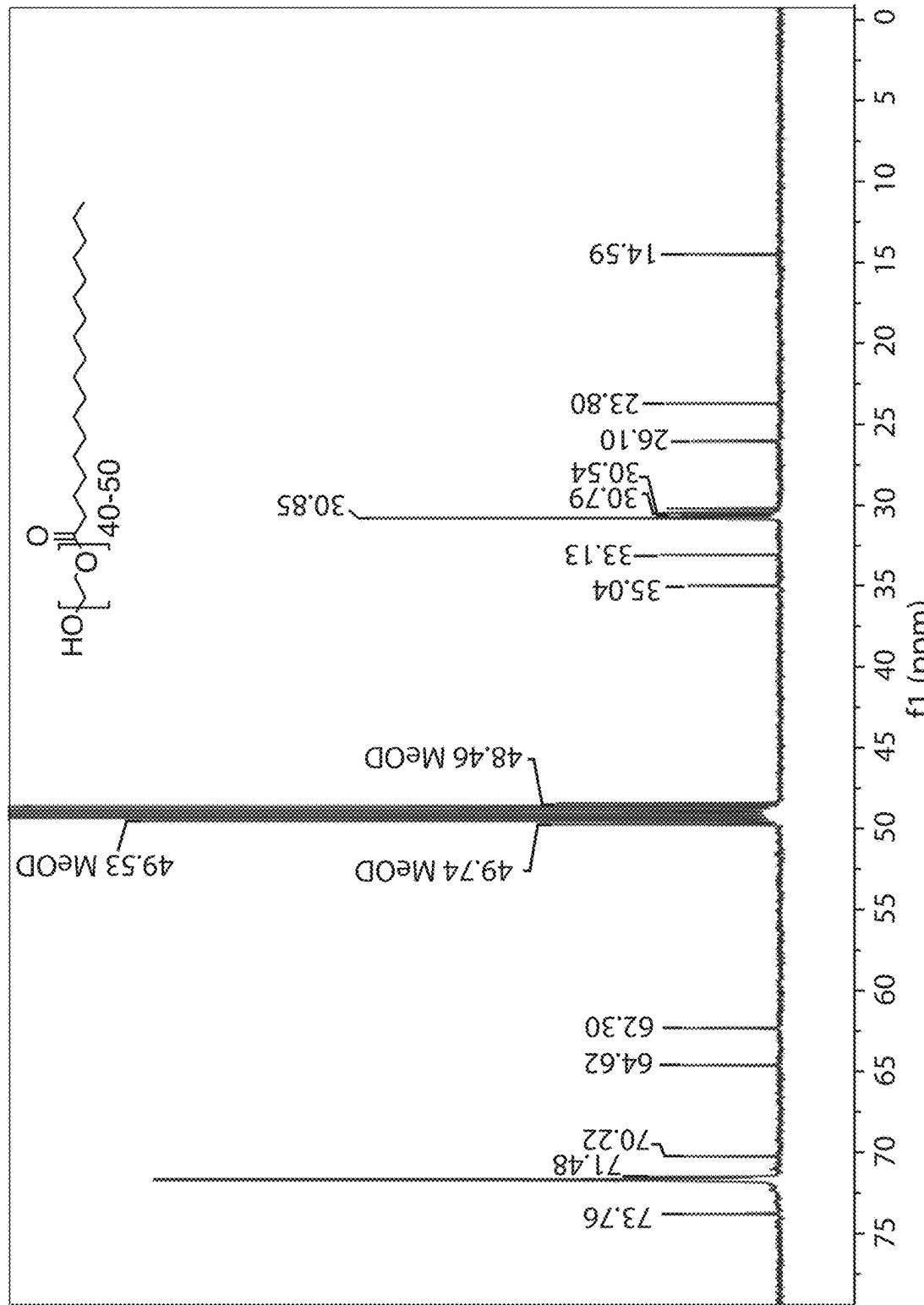


Figure 12C

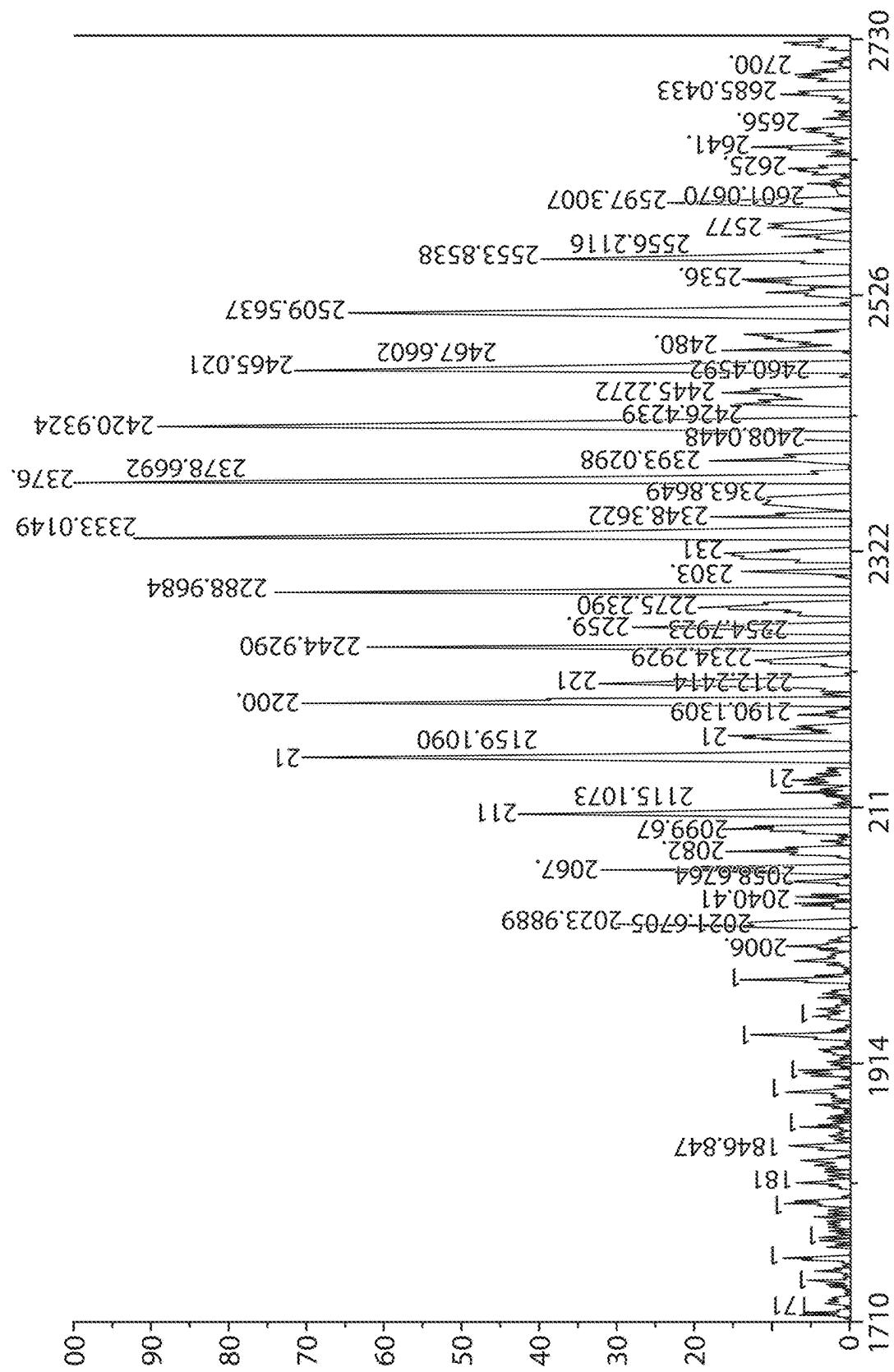


Figure 12D

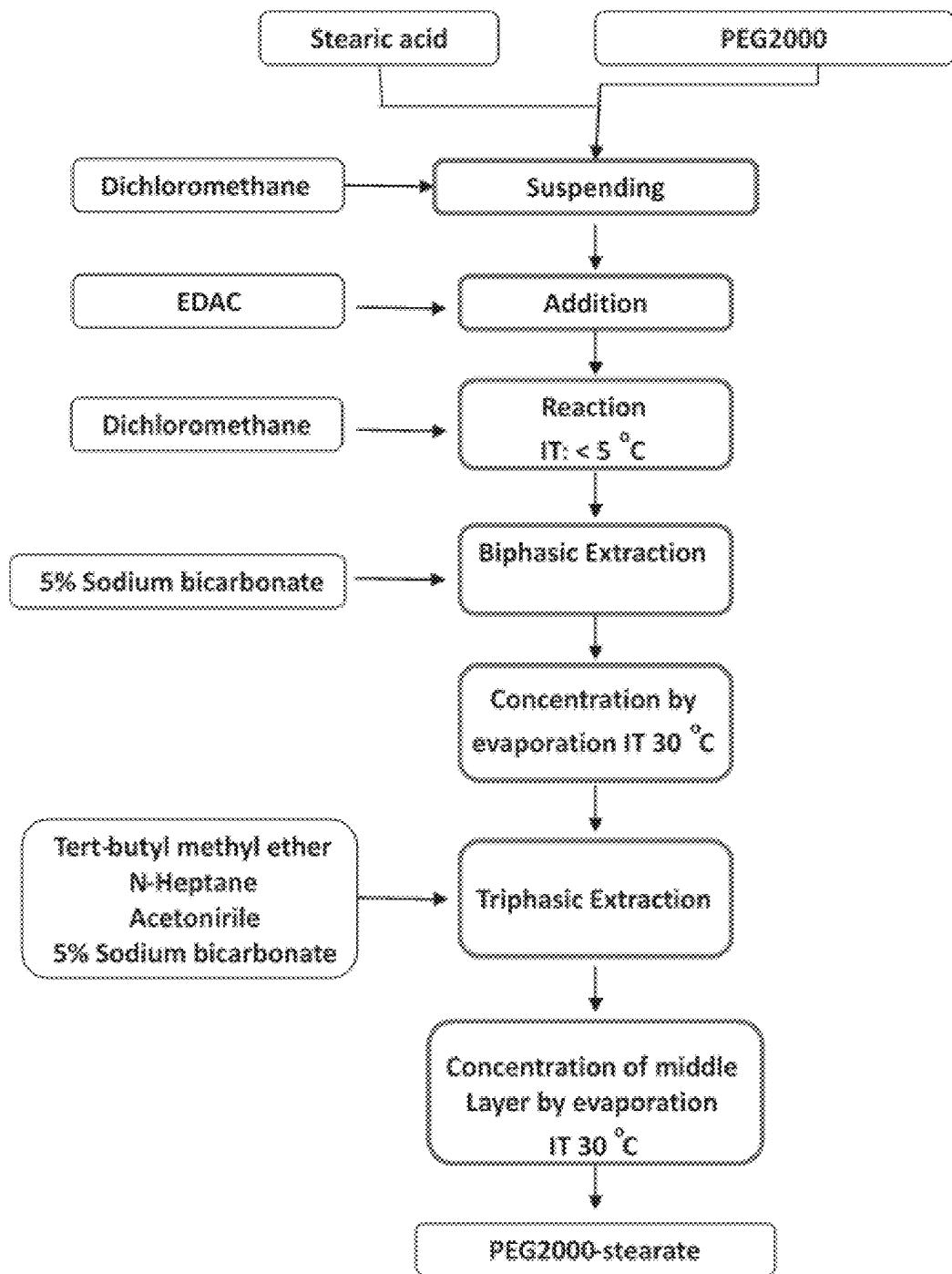


Figure 13

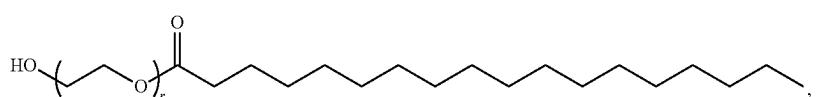
HIGH-PURITY PEG LIPIDS AND USES THEREOF

RELATED APPLICATIONS

This application is a national stage filing under 35 U.S.C. § 371 of International PCT Application, PCT/US2019/051906, filed Sep. 19, 2019, which claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Applications,

respect to the PEG lipids. The PEG lipids can be part of a composition or formulation, and the composition or formulation may comprise other components. Several non-limiting examples of PEG lipids are provided herein.

For example, in certain embodiments, the plurality of PEG lipids is a plurality of compounds of Formula (I), or salts thereof. Provided herein is a plurality of compounds of Formula (I):



U.S. Ser. No. 62/733,456, filed Sep. 19, 2018; and U.S. Ser. No. 62/745,164, filed Oct. 12, 2018, the entire contents of each of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Lipids comprising polyethylene glycol units (PEG lipids) have wide ranging applications in, for example, consumer products, pharmaceutical formulations, cosmetic compositions, and drug delivery. In particular, PEG lipids are useful as components in lipid nanoparticles (LNPs) for the encapsulation and delivery of agents (e.g., therapeutic agents).

Effective in vivo delivery of active agents such as small molecule drugs, proteins, peptides, and nucleic acids represents a continuing medical challenge. Some agents and/or particles are recognized by the immune system, resulting in decreased efficacy. To address this issue, certain formulations have incorporated polymers such as PEG lipids, which are thought to cloak or mask the agent, thereby reducing its antigenicity and immunogenicity. However, even these “stealth” formulations have their limitations, including an inability to be repeatedly and frequently dosed, for example, over a period of days without the loss of activity. In addition, some agents or formulations when administered in vivo may interact with one or more cells or factors, potentially interfering with their functions, and ultimately resulting in adverse effects. Such adverse effects may limit the administration frequency and/or administered dose of the agent, or may preclude in vivo use altogether.

Formulating LNPs to achieve ideal physical characteristics and bioperformance is challenging. Several factors dictate LNP properties and performance, including choice and ratio of the lipid components, ratio of lipid components to the encapsulated agent, and more.

SUMMARY OF THE INVENTION

The present disclosure is based, at least in part, on the discovery that high-purity PEG lipids exhibit superior physical and biological properties, particularly when used in lipid nanoparticle (LNP) formulations. Therefore, the present disclosure provides PEG lipids at a recommended purity (at least 87% pure), e.g., for use in formulations, such as LNP formulations.

Provided herein are pluralities of PEG lipids (i.e., mixtures, batches, solutions, etc. of PEG lipids) having a purity of 87% or greater with respect to the PEG lipids (also referred to herein as “high-purity PEG lipids”). When “PEG lipids” or “high-purity PEG lipids” are referred to herein, the PEG lipids have the proscribed purity of 87% or greater with

or salts thereof, wherein: r is independently an integer from 35-55, inclusive; and the plurality of compounds has a purity of 87% or greater with respect to compounds of Formula (I).

A plurality of PEG lipids provided herein may comprise other components such as solvents, carriers, excipients, etc., and can therefore be in the form of a solution or composition. When the PEG lipids are part of a solution or composition, it is to be understood that the proscribed/recommended purity (e.g., of 87% or greater) is with respect to the PEG lipids component of the solution or composition.

The high-purity PEG lipids provided herein have application in pharmaceutical compositions, cosmetic compositions, and drug delivery systems, including lipid nanoparticle (LNP) formulations. Therefore, provided herein are LNPs comprising a plurality of PEG lipids provided herein (i.e., a plurality of PEG lipids with a purity of 87% or greater with respect to the PEG lipids, e.g., a plurality of compounds of Formula (I) with a purity of 87% or greater with respect to the compounds of Formula (I)).

In addition to high-purity PEG lipids, any formulation or composition described herein (e.g., an LNP formulations) may comprise lipids other than PEG lipids. In certain embodiments, an LNP provided herein further comprises an ionizable amino lipid, a helper lipid, and/or a structural lipid. In certain embodiments, an LNP provided herein comprises a plurality of PEG lipids provided herein (e.g., a plurality of compounds of Formula (I) provided herein), an ionizable amino lipid, a helper lipid, and a structural lipid. Several non-limiting examples of PEG lipids, ionizable amino lipids, helper lipids, and structural lipids are provided herein. The LNPs provided herein are useful for the delivery of therapeutic agents and therefore may comprise (e.g., encapsulate) one or more agents (e.g., therapeutic agents).

The PEG lipids and LNPs described herein may have improved physical and biological properties as compared to state-of-the-art formulations. It is common for lipid nanoparticles (LNPs) to induce an innate immune response after administration to a subject. In some instances, components of LNPs, such as phosphatidylcholines, may induce the production of natural IgM and/or IgG molecules, which may be mediated by activation of B1 cells, such as B1a and/or B1b cells. These biological mechanisms may contribute to drug responses caused by LNPs, including accelerated blood clearance (ABC) and dose-limiting toxicity such as acute phase response (APR), and complement activation-related pseudoallergy (CARPA). LNP formulations have been developed that are less susceptible to recognition, and thus clearance (e.g., ABC), by the immune system. Some state-of-the-art LNP formulations have improved clearance

profiles, and in some instances, improved toxicity profiles. For some examples of LNP formulations that are less susceptible to blood clearance, see International Publication No. WO 2017/099823, published Jun. 15, 2017, the entire contents of which is incorporated herein by reference.

The present disclosure in part relates to the discovery that LNPs comprising high-purity PEG lipids have improved physical and biological properties, including being less susceptible to recognition and clearance by the body. For example, in certain embodiments, a LNP formulation described herein (i.e., comprising a plurality of PEG lipids at the recommended purity of 87% or greater) is less susceptible to accelerated blood clearance (ABC) as compared to an LNP formulation comprising a plurality of PEG lipids with a purity of less than 87%. For example, in certain embodiments, an LNP formulation comprising a plurality of compounds of Formula (I) as described herein (i.e., at the recommended purity of 87% or greater) is less sensitive to accelerated blood clearance (ABC) as compared to an LNP formulation comprising a plurality of compounds of Formula (I) with a purity of less than 87%. LNP formulations comprising high purity PEG lipids described herein may also have decreased B cell binding (e.g., B1a and/or B1b cells) and/or decreased IgM binding as compared with less pure formulations.

The present disclosure also provides pharmaceutical and cosmetic compositions comprising the high-purity PEG lipids described herein. In another aspect, the present disclosure provides compositions comprising a plurality of LNPs described herein.

In another aspect, the present disclosure provides methods for delivering one or more therapeutic agents to a subject, the methods comprising administering to the subject an LNP provided herein, wherein the LNP encapsulates the one or more therapeutic agents of interest.

In some instances, a method for delivering one or more therapeutic agents may comprise:

- (i) administering a first dose of an agent to a subject,
- (ii) administering a second or subsequent dose of the agent to the subject, wherein the second or subsequent dose is administered within 2 weeks of the first or prior dose, and
- (iii) repeating step (ii) one or more times.

In certain embodiments, the agent is formulated with an LNP that does not promote ABC. As described herein, such an LNP can comprise the high-purity PEG lipids described herein. In certain embodiments, the half-life of the agent after the second and subsequent dose is at least 50% or more of the half-life of the agent after the first dose. In certain embodiments, the activity or blood concentration of the agent after the second and subsequent dose is at least 50% or more of the activity or blood concentration of the agent after the first dose.

In yet another aspect, provided herein are methods for preparing the PEG lipids provided herein (e.g., compounds of Formula (I)).

The details of certain embodiments of the invention are set forth in the Detailed Description of Certain Embodiments, as described below. Other features, objects, and advantages of the invention will be apparent from the Definitions, Examples, Figures, and Claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which constitute a part of this specification, illustrate several embodiments of the

invention and together with the description, serve to explain the principles of the invention.

FIG. 1. Structures of PEG Lipids of Formula (I) (also known as HO-PEG-Stearate, PEG-Stearate, Compound (I)) and associated impurities.

FIG. 2. Dynamic Light Scattering (DLS) profiles of LNPs formulated with PEG lipids of various purities (Lot No. 5: 97.8% purity; Corden: 98.5% purity; BioVectra: 86.6% purity; Sigma: 43.8% purity). DLS shows much sharper particle size distributions (PSD) for LNPs formulated with higher purity PEG lipids.

FIG. 3. Ultra Performance-Liquid Chromatography-Charged Aerosol Detection (UPLC-CAD) chromatograms of lipids in LNPs (HO-PEG-Stearate, Compound (B), Cholesterol, DSPE).

FIG. 4. Heparin-sepharose column binding of LNP formulations with varying purities of PEG lipids.

FIG. 5. PEG-shedding of each LNP formulation was found to be the same. For each formulation, all PEG is shed at the first time point (30 minutes).

FIG. 6. Measured amounts of accessible mRNA using the Bloody Escape of Encapsulation by Fluorescence In Vitro (BEEFI) assay. Formulations using higher purity compounds of Formula (I) showed greater amounts of accessible mRNA by Ribogreen detection.

FIG. 7. PSD of LNP formulation using highly impure PEG lipids of Formula (I). LNPs were formulated according to Table 7 and Table 8 (see Examples).

FIG. 8. PSD of formulated fluorescent-tagged LNPs with varying purities of PEG lipids. *One addition of Sigma Compound (I), ~0.6-0.7 mol % Compound (I) in final product. **Two additions of Sigma Compound (I), ~1.3-1.4 mol % Compound (I) in final product.

FIG. 9. ApoE binding assay with LNPs comprising varying purities of PEG lipids. *One addition of Sigma Compound (I), ~0.6-0.7 mol % Compound (I) in final product. **Two additions of Sigma Compound (I), ~1.3-1.4 mol % Compound (I) in final product.

FIG. 10. IgM assay with LNPs comprising varying purities of PEG lipids. The signal measured in naïve serum indicates binding of natural IgM arising from the PC epitope. *One addition of Sigma Compound (I), ~0.6-0.7 mol % Compound (I) in final product. **Two additions of Sigma Compound (I), ~1.3-1.4 mol % Compound (I) in final product. LNPs comprising higher purity PEG lipids exhibit significantly less binding to IgM as compared to less pure formulations.

FIG. 11. Exemplary synthesis of Compound (I).

FIG. 12A. IR Spectrum of Compound (I). FIG. 12B. Proton (H) NMR Spectrum of Compound (I) in Deuterated Chloroform (CDCl_3). FIG. 12C. Carbon (^{13}C) NMR Spectrum of Compound (I) in Deuterated Chloroform (CDCl_3). FIG. 12D. MALDI-TOF Scan Mass Spectrum of Compound (I).

FIG. 13 shows a flowchart outlining an exemplary synthesis/purification method for the preparation of a compound of Formula (I) (PEG2000 stearate).

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

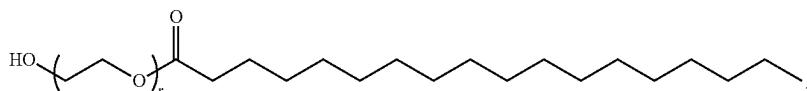
The present disclosure in part provides PEG lipids at a specified purity ("high-purity PEG lipids"), e.g., for use in pharmaceutical composition, cosmetic compositions, and drug delivery systems, e.g., for use in lipid nanoparticle (LNP) formulations. The present disclosure also provides LNPs comprising the high-purity PEG lipids described

herein, and methods of using the same. For example, the LNPs provided herein are useful for the delivery of an agent (e.g., therapeutic agent) to a subject. In certain embodiments, the LNPs provided herein are less susceptible, if at all susceptible, to accelerated blood clearance (ABC) after administration to a subject.

High Purity PEG Lipids

Provided herein are pluralities of PEG lipids, wherein a plurality of PEG lipids has a purity of 87% or greater with respect to the PEG lipids. When "PEG lipids" are referred to herein (also referred to herein as "high-purity PEG lipids"), it is implied that the PEG lipid mixture has the proscribed/recommended purity of 87% or greater with respect to the PEG lipids. In certain embodiments, the PEG lipids in the plurality of PEG lipids are the same PEG lipids. In certain embodiment, the plurality of PEG lipids comprises a mixture of PEG lipids. PEG lipids are any lipids that comprise a PEG (polyethylene glycol) group. Non-limiting examples of PEG lipids are provided herein.

For example, in certain embodiments, a PEG lipid is a compound of Formula (I):



90-95%, inclusive. In certain embodiments, the purity is from 95-100%, inclusive. In certain embodiments, the purity is from 95-99.9%, inclusive.

In certain embodiments, the purity is from 90-100%, inclusive. In certain embodiments, the purity is from 90-99.9%, inclusive. In certain embodiments, the purity is from 91-99.9%, inclusive. In certain embodiments, the purity is from 92-99.9%, inclusive. In certain embodiments, the purity is from 93-99.9%, inclusive. In certain embodiments, the purity is from 94-99.9%, inclusive. In certain embodiments, the purity is from 95-99.9%, inclusive. In certain embodiments, the purity is from 96-99.9%, inclusive. In certain embodiments, the purity is from 97-99.9%, inclusive. In certain embodiments, the purity is from 98-99.9%, inclusive. In certain embodiments, the purity is from 99-99.9%, inclusive.

In certain embodiments, the plurality of PEG lipids has a purity of approximately 87% with respect to the PEG lipids. In certain embodiments, the purity is approximately 87.5%. In certain embodiments, the purity is approximately 88%. In certain embodiments, the purity is approximately 88.5%. In

or a salt thereof; wherein r is independently an integer from 35-55, inclusive.

A plurality of PEG lipids provided herein may comprise other components such as solvents, carriers, excipients, etc., and can therefore be in the form of a solution or composition. When the PEG lipids are part of a solution or composition, it is to be understood that the proscribed/recommended purity (e.g., of 87% or greater) is with respect to the PEG lipids component of the solution or composition.

As described herein, the plurality of PEG lipids has a purity greater than 87% with respect to the PEG lipids. In certain embodiments, the purity is greater than 90%. In certain embodiments, the purity is greater than 91%. In certain embodiments, the purity is greater than 92%. In certain embodiments, the purity is greater than 93%. In certain embodiments, the purity is greater than 94%. In certain embodiments, the purity is greater than 95%. In certain embodiments, the purity is greater than 96%. In certain embodiments, the purity is greater than 97%. In certain embodiments, the purity is greater than 98%. In certain embodiments, the purity is greater than 98.5%. In certain embodiments, the purity is greater than 99%. In certain embodiments, the purity is greater than 99.1%. In certain embodiments, the purity is greater than 99.2%. In certain embodiments, the purity is greater than 99.3%. In certain embodiments, the purity is greater than 99.4%. In certain embodiments, the purity is greater than 99.5%. In certain embodiments, the purity is greater than 99.6%. In certain embodiments, the purity is greater than 99.7%. In certain embodiments, the purity is greater than 99.8%. In certain embodiments, the purity is greater than 99.9%. In certain embodiments, the purity is 100%.

In certain embodiments, the plurality of PEG lipids has a purity from 87-100%, inclusive, with respect to the PEG lipids. In certain embodiments, the purity is from 87-99.9%, inclusive.

In certain embodiments, the purity is from 87-90%, inclusive. In certain embodiments, the purity is from

30 certain embodiments, the purity is approximately 89%. In certain embodiments, the purity is approximately 89.5%. In certain embodiments, the purity is approximately 90%. In certain embodiments, the purity is approximately 90.5%. In certain embodiments, the purity is approximately 91%. In certain embodiments, the purity is approximately 91.5%. In certain embodiments, the purity is approximately 92%. In certain embodiments, the purity is approximately 92.5%. In certain embodiments, the purity is approximately 93%. In certain embodiments, the purity is approximately 93.5%. In certain embodiments, the purity is approximately 94%. In certain embodiments, the purity is approximately 94.5%. In certain embodiments, the purity is approximately 95%. In certain embodiments, the purity is approximately 95.5%. In certain embodiments, the purity is approximately 96%. In certain embodiments, the purity is approximately 96.6%. In certain embodiments, the purity is approximately 97%. In certain embodiments, the purity is approximately 97.7%. In certain embodiments, the purity is approximately 98%. In certain embodiments, the purity is approximately 98.5%. In certain embodiments, the purity is approximately 99%. In certain embodiments, the purity is approximately 99.5%. In certain embodiments, the purity is approximately 99.9%. In certain embodiments, the purity is approximately 100%. In certain embodiments, the purity is approximately 98.1%, 98.2%, 98.3%, 98.4%, 98.5%, 98.6%, 98.7%, 98.8%, or 98.9%. In certain embodiments, the purity is approximately 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%.

60 In certain embodiments, the plurality of PEG lipids consists essentially of PEG lipids.

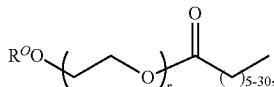
In certain embodiments, the plurality of PEG lipids is substantially free of other compounds. In certain embodiments, the plurality of PEG lipids is substantially free of impurities. The term "impurities" refers to compounds other than the PEG lipids. In certain embodiments, the plurality of PEG lipids comprises less than 13% of total impurities. In

certain embodiments, the plurality of PEG lipids comprises less than 12% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 11% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 10% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 9% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 8% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 7% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 6% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 5% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 4% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 3% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 2% of total impurities. In certain embodiments, the plurality of PEG lipids comprises less than 1% of total impurities.

In certain embodiments, the impurities include one or more fatty acids. In certain embodiments, the impurities include one or more PEG polymers. In certain embodiments, the impurities include one or more fatty acids and one or more PEG polymers.

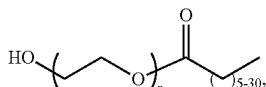
In certain embodiments, the plurality of PEG lipids is substantially free of PEG. In certain embodiments, the plurality of PEG lipids is substantially free of fatty acids. "Substantially free," when used herein in regard to an impurity, refers to a maximum of 0-5% of impurity present. In certain embodiments, 0-2% of impurity may be present. In certain embodiments, 0-1% of impurity may be present.

In certain embodiments, the PEG lipid is compound of the following formula:



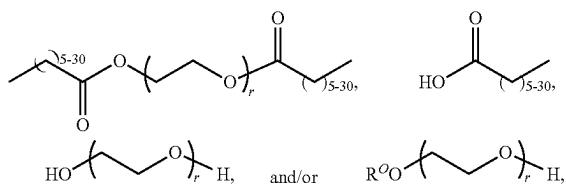
or a salt thereof, wherein r is independently an integer from 35-55, inclusive; and R^O is hydrogen, optionally substituted alkyl, or an oxygen protecting group.

In certain embodiments, the PEG lipid is compound of the following formula:



or a salt thereof, wherein r is independently an integer from 35-55, inclusive.

In certain embodiments, the impurities are one or more of the following:



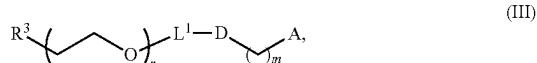
or salts thereof, wherein r is independently an integer from 35-55, inclusive; and R^O is hydrogen, optionally substituted alkyl, or an oxygen protecting group.

As described herein, the present invention relates in part to high-purity PEG lipids. Such species may be alternately referred to as PEGylated lipids. Non-limiting examples of PEG lipids are provided herein. For other embodiments, including further examples of PEG lipids useful in the present invention, see International Publication No. WO 2017/099823, published Jun. 15, 2017, the entire contents of which is incorporated herein by reference. In some embodiments, PEG lipids useful in the present invention can be PEGylated lipids described in International Publication No. WO 2012/099755, published Jul. 26, 2012, the contents of which is herein incorporated by reference in its entirety.

A PEG lipid is a lipid modified with polyethylene glycol. A PEG lipid may be selected from the non-limiting group consisting of PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, and mixtures thereof. For example, a PEG lipid may be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, or a PEG-DSPE lipid. In some embodiments the PEG-modified lipids are a modified form of PEG-DMG.

Any of these exemplary PEG lipids described herein may be modified to comprise a hydroxyl group on the PEG chain. In certain embodiments, the PEG lipid is a PEG-OH lipid. As generally defined herein, a "PEG-OH lipid" (also referred to herein as "hydroxy-PEGylated lipid") is a PEGylated lipid having one or more hydroxyl (—OH) groups on the lipid. In certain embodiments, the PEG-OH lipid includes one or more hydroxyl groups on the PEG chain. In certain embodiments, a PEG-OH or hydroxy-PEGylated lipid comprises an —OH group at the terminus of the PEG chain. Each possibility represents a separate embodiment of the present invention.

In certain embodiments, a PEG lipid useful in the present invention is a compound of Formula (III). Provided herein are compounds of Formula (III):



or salts thereof, wherein:

R³ is —OR^O;
R^O is hydrogen, optionally substituted alkyl, or an oxygen protecting group;

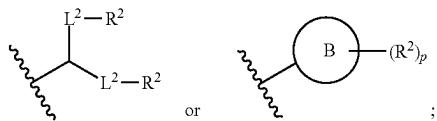
r is an integer between 1 and 100, inclusive;

L¹ is optionally substituted C₁₋₁₀ alkylene, wherein at least one methylene of the optionally substituted C₁₋₁₀ alkylene is independently replaced with optionally substituted carbocyclene, optionally substituted heterocyclene, optionally substituted arylene, optionally substituted heteroarylene, —O—, —N(R^N)—, —S—, —C(O)—, —C(O)N(R^N)—, —NR^NC(O)—, —C(O)O—, —OC(O)O—, —OC(O)N(R^N)—, —NR^NC(O)O—, or —NR^NC(O)N(R^N)—;

D is a moiety obtained by click chemistry or a moiety cleavable under physiological conditions;

m is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

A is of the formula:



5

or a salt thereof.

each instance of L² is independently a bond or optionally substituted C₁₋₆ alkylene, wherein one methylene unit of the optionally substituted C₁₋₆ alkylene is optionally replaced with —O—, —N(R^N)—, —S—, —C(O)—, —C(O)N(R^N)—, —NR^NC(O)—, —C(O)O—, —OC(O)—, —OC(O)O—, —OC(O)N(R^N)—, —NR^NC(O)O—, or —NR^NC(O)N(R^N)—;

each instance of R² is independently optionally substituted C₁₋₃₀ alkyl, optionally substituted C₁₋₃₀ alkenyl, or optionally substituted C₁₋₃₀ alkynyl; optionally wherein one or more methylene units of R² are independently replaced with optionally substituted carbocyclyene, optionally substituted heterocyclylene, optionally substituted arylene, optionally substituted heteroarylene, —N(R^N)—, —O—, —S—, —C(O)—, —C(O)N(R^N)—, —NR^NC(O)—, —NR^NC(O)N(R^N)—, —C(O)O—, —OC(O)—, —OC(O)O—, —OC(O)N(R^N)—, —NR^NC(O)O—, —C(O)S—, —SC(O)—, —C(=NR^N)—, —C(=NR^N)N(R^N)—, —NR^NC(=NR^N)—, —NR^NC(=NR^N)N(R^N)—, —C(S)—, —C(S)N(R^N)—, —NR^NC(S)—, —NR^NC(S)N(R^N)—, —S(O)—, —OS(O)—, —S(O)O—, —OS(O)O—, —OS(O)₂—, —S(O)₂O—, —OS(O)₂O—, —N(R^N)S(O)O—, —S(O)N(R^N)—, —N(R^N)S(O)N(R^N)—, —OS(O)N(R^N)—, —N(R^N)S(O)O—, —S(O)₂—, —N(R^N)S(O)₂—, —S(O)₂N(R^N)—, —N(R^N)S(O)₂N(R^N)—, —OS(O)₂N(R^N)—, or —N(R^N)S(O)₂O—;

each instance of R^N is independently hydrogen, optionally substituted alkyl, or a nitrogen protecting group;

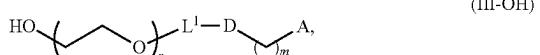
Ring B is optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl, or optionally substituted heteroaryl; and

p is 1 or 2.

45

In certain embodiments, the compound of Formula (III) is a PEG-OH lipid (i.e., R³ is —OR^O, and R^O is hydrogen). In certain embodiments, the compound of Formula (III) is of Formula (III-OH):

50

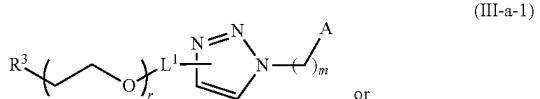


55

or a salt thereof.

In certain embodiments, D is a moiety obtained by click chemistry (e.g., triazole). In certain embodiments, the compound of Formula (III) is of Formula (III-a-1) or (III-a-2):

60

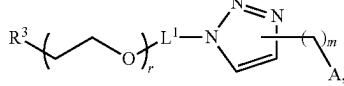


65

10

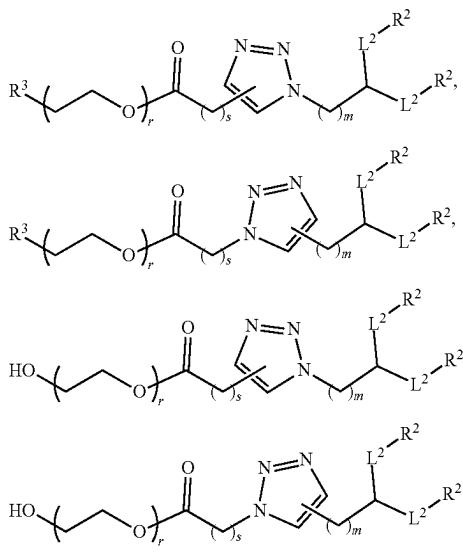
-continued

(III-a-2)



or a salt thereof.

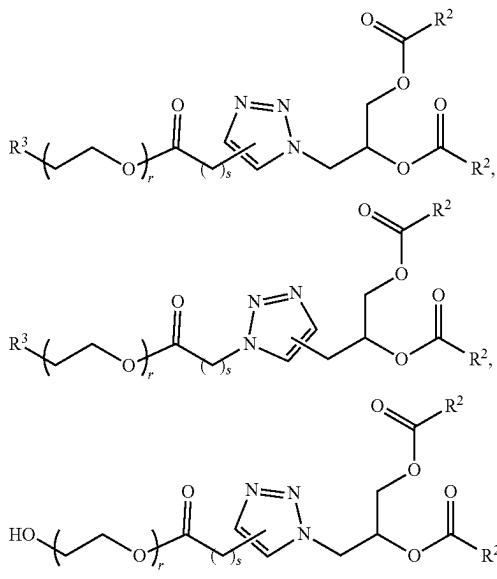
In certain embodiments, the compound of Formula (III) is of one of the following formulae:



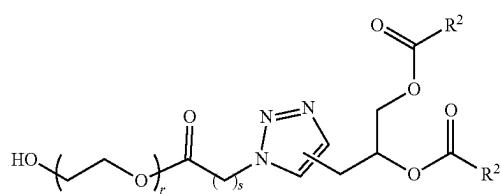
or a salt thereof, wherein

s is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

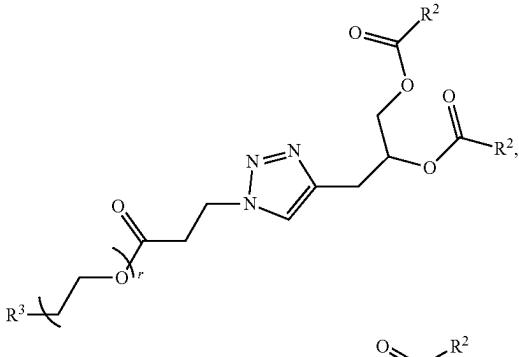
In certain embodiments, the compound of Formula (III) is of one of the following formulae:



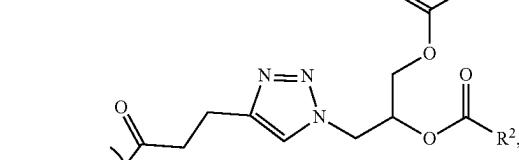
11
-continued



12
-continued

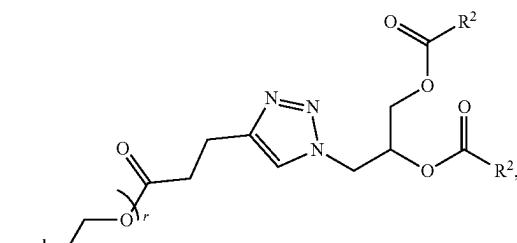


15

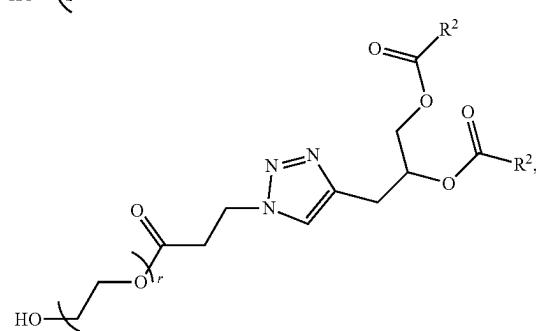


or a salt thereof.

In certain embodiments, a compound of Formula (III) is
of one of the following formulae:

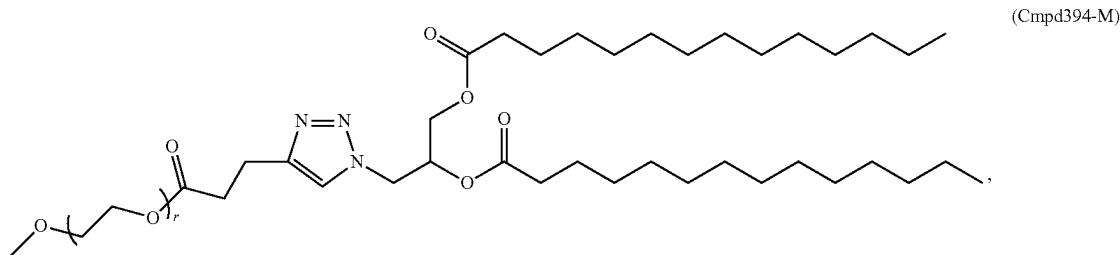
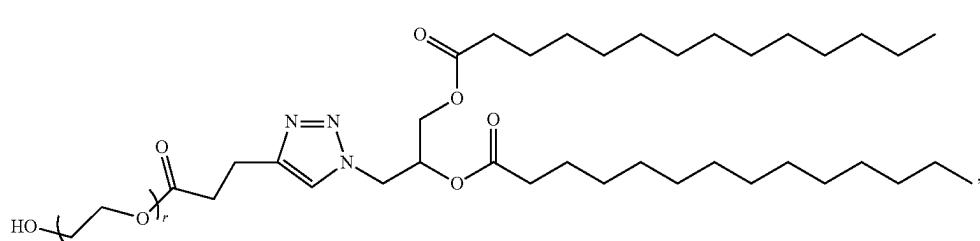


30



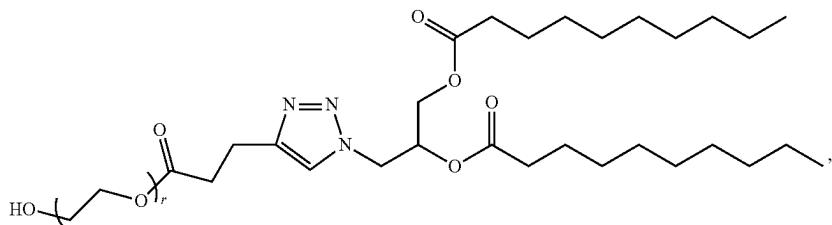
or a salt thereof.

In certain embodiments, a compound of Formula (III) is
of one of the following formulae:

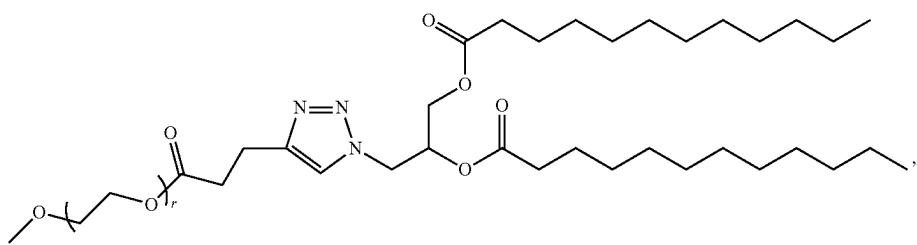


-continued

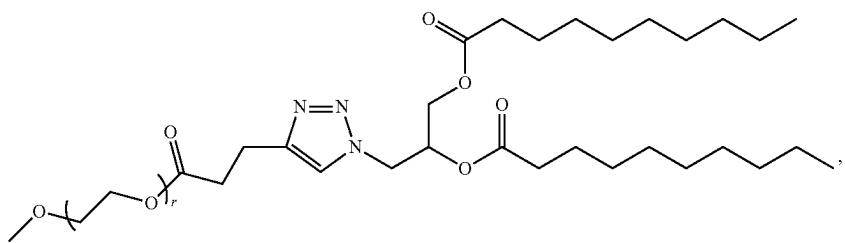
(Cmpd396)



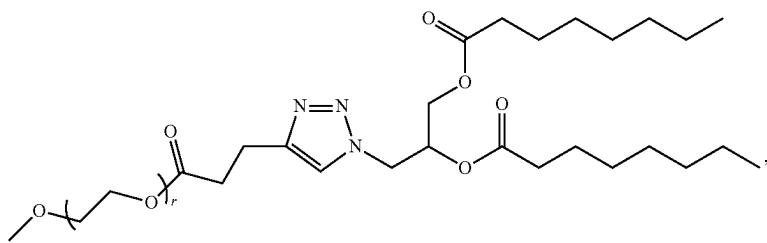
(Cmpd396A)



(Cmpd395)

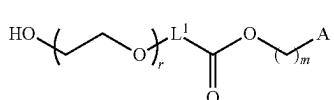


(Cmpd397)

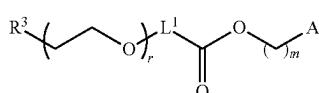


or a salt thereof.

In certain embodiments, D is a moiety cleavable under physiological conditions (e.g., ester, amide, carbonate, carbamate, urea). In certain embodiments, a compound of Formula (III) is of Formula (III-b-1) or (III-b-2):



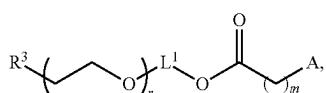
(III-b-1-OH)



(III-b-2-OH)

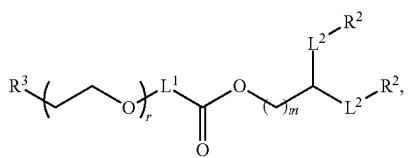
55 or a salt thereof.

In certain embodiments, the compound of Formula (III) is of one of the following formulae:



(III-b-2)

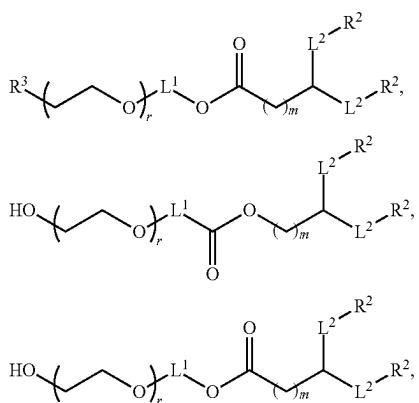
60



or a salt thereof.

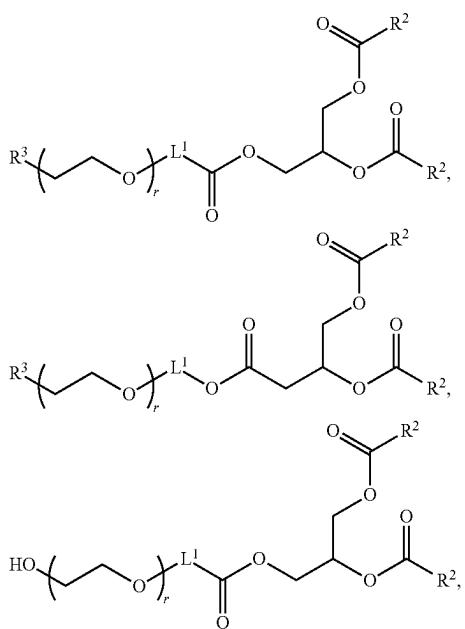
In certain embodiments, a compound of Formula (III) is of Formula (III-b-1-OH) or (III-b-2-OH):

15
-continued

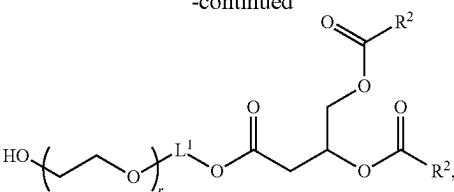


or a salt thereof.

In certain embodiments, a compound of Formula (III) is of one of the following formulae:

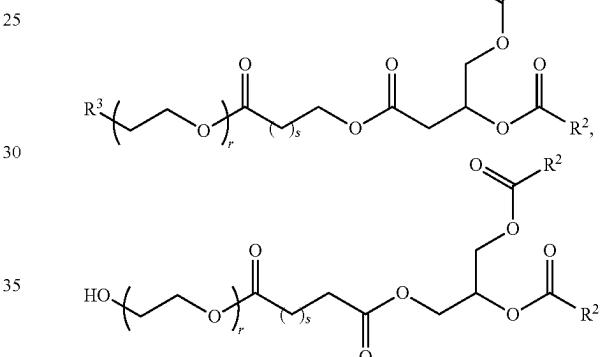
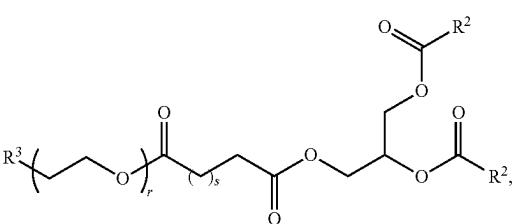


16
-continued



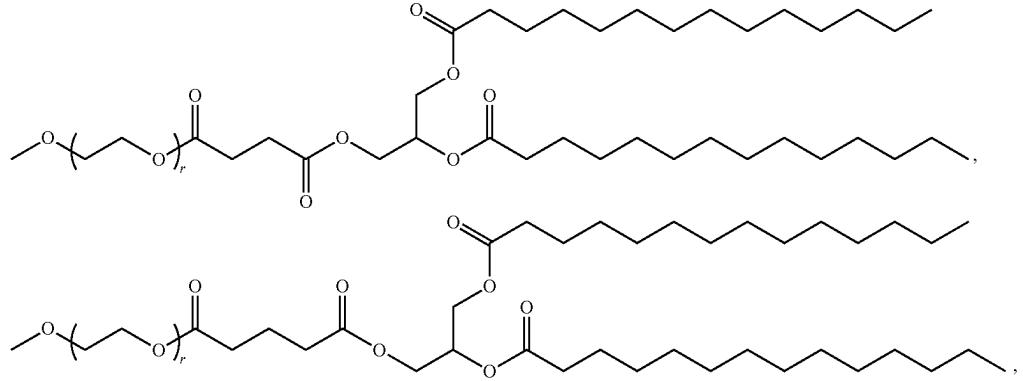
or a salt thereof.

In certain embodiments, a compound of Formula (III) is of one of the following formulae:



or a salt thereof.

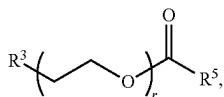
In certain embodiments, a compound of Formula (III) is of one of the following formulae:



or salts thereof.

17

In certain embodiments, a PEG lipid useful in the present invention is a PEGylated fatty acid. In certain embodiments, a PEG lipid useful in the present invention is a compound of Formula (V). Provided herein are compounds of Formula (V):



(V)

or a salts thereof, wherein:

R³ is —OR^O;

R^O is hydrogen, optionally substituted alkyl or an oxygen protecting group;

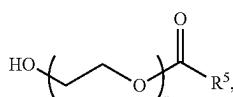
r is an integer between 1 and 100, inclusive;

R⁵ is optionally substituted C₁₀₋₄₀ alkyl, optionally substituted C₁₀₋₄₀ alkenyl, or optionally substituted C₁₀₋₄₀ alkynyl; and optionally one or more methylene groups of R are replaced with optionally substituted carbocyclene, optionally substituted heterocyclene, optionally substituted arylene, optionally substituted heteroarylene, —N(R^NC(O)—, —C(O)N(R^N)—, —NR^NC(O)—, —NR^NC(O)N

(R^N)—, —C(O)O—, —OC(O)—, —OC(O)O—, —OC(O)N(R^N)—, —NR^NC(O)O—, —C(O)S—, —SC(O)—, —C(=NR^N)—, —C(=NR^N)N(R^N)—, —NR^NC(=NR^N)N(R^N)—, —C(S)—, —C(S)N(R^N)—, —NR^NC(S)—, —NR^NC(S)N(R^N)—, —S(O)—, —OS(O)—, —SO(O)—, —OS(O)O—, —OS(O)₂—, —S(O)₂O—, —OS(O)₂O—, —N(R^N)S(O)—, —S(O)N(R^N)—, —N(R^N)S(O)N(R^N)—, —OS(O)N(R^N)—, —N(R^N)S(O)O—, —S(O)₂—, —N(R^N)S(O)₂—, —S(O)₂N(R^N)—, —N(R^N)S(O)₂N(R^N)—, —OS(O)₂N(R^N)—, or —N(R^N)S(O)₂O—; and

each instance of R^N is independently hydrogen, optionally substituted alkyl, or a nitrogen protecting group.

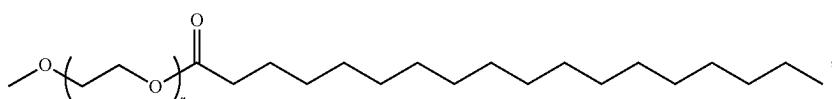
In certain embodiments, the compound of Formula (V) is of Formula (V-OH):



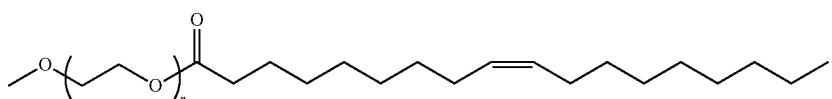
(V-OH)

or a salt thereof.

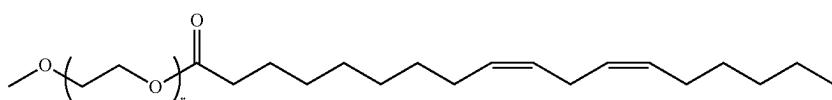
In certain embodiments, a compound of Formula (V) is of one of the following formulae:



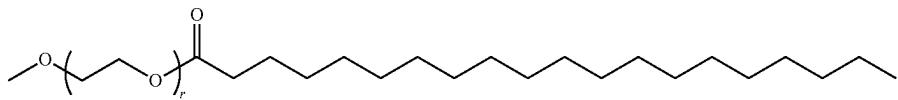
(Cmpd400)



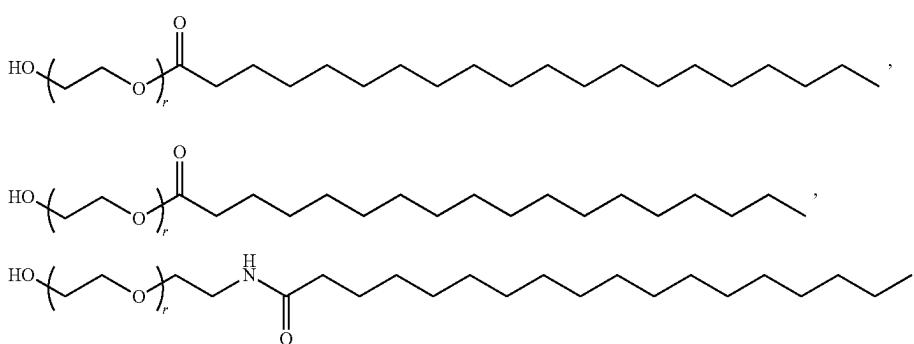
(Cmpd401)



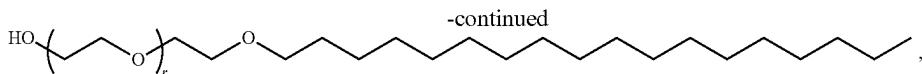
(Cmpd402)



(I)



19

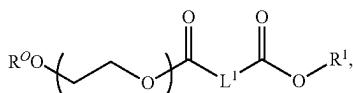


20

5

or a salt thereof.

In certain embodiments, PEG lipids useful in the present invention are PEG lipids of Formula (L-I):



10

or pharmaceutically acceptable salts thereof; wherein:

L¹ is a bond, optionally substituted C₁₋₃ alkylene, optionally substituted C₁₋₃ heteroalkylene, optionally substituted C₂₋₃ alkenylene, optionally substituted C₂₋₃ alkynylene;

20

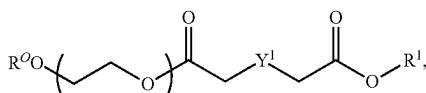
R¹ is optionally substituted C₅₋₃₀ alkyl, optionally substituted C₅₋₃₀ alkenyl, or optionally substituted C₅₋₃₀ alkynyl;

R^O is hydrogen, optionally substituted alkyl, optionally substituted acyl, or an oxygen protecting group; and

25

r is an integer from 2 to 100, inclusive.

In certain embodiments, the PEG lipid of Formula (L-I) is of the following formula:



30

or a pharmaceutically acceptable salt thereof; wherein:

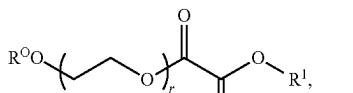
Y¹ is a bond, —CR₂—, —O—, —NR^N—, or —S—;

40

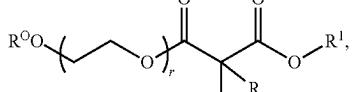
each instance of R is independently hydrogen, halogen, or optionally substituted alkyl; and

R^N is hydrogen, optionally substituted alkyl, optionally substituted acyl, or a nitrogen protecting group.

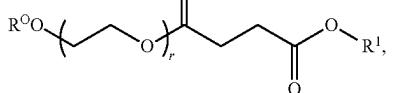
In certain embodiments, the PEG lipid of Formula (L-I) is of one of the following formulae:



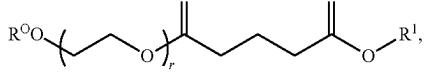
50



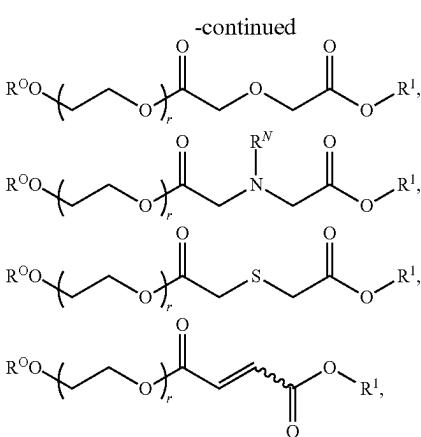
55



60



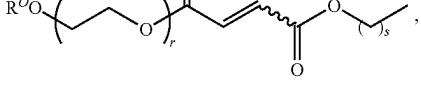
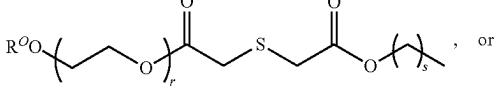
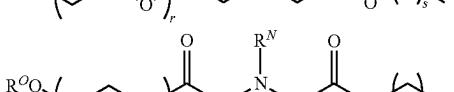
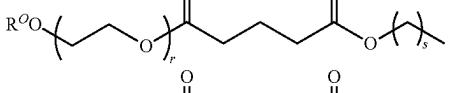
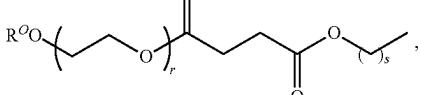
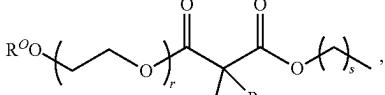
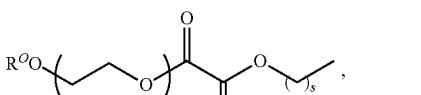
65



or a pharmaceutically acceptable salt thereof; wherein:

each instance of R is independently hydrogen, halogen, or optionally substituted alkyl.

In certain embodiments, the PEG lipid of Formula (L-I) is of one of the following formulae:

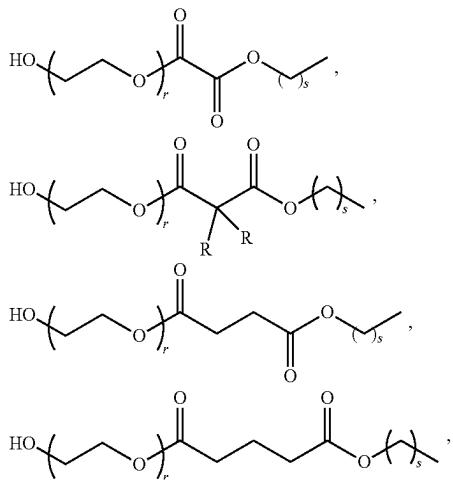


21

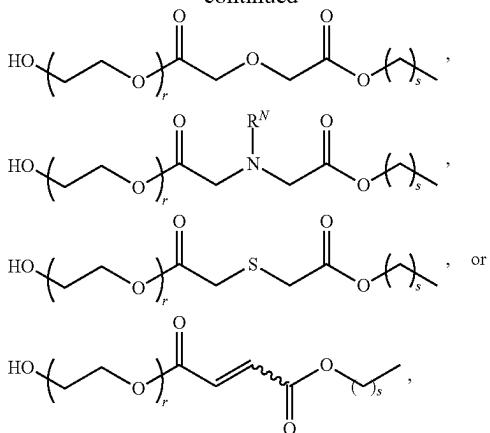
or a pharmaceutically acceptable salt thereof, wherein:

 s is an integer from 5-25, inclusive.

In certain embodiments, the PEG lipid of Formula (L-I) is of one of the following formulae:

**22**

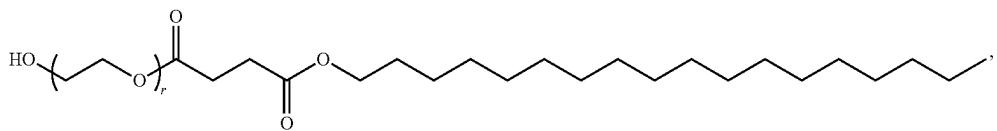
-continued



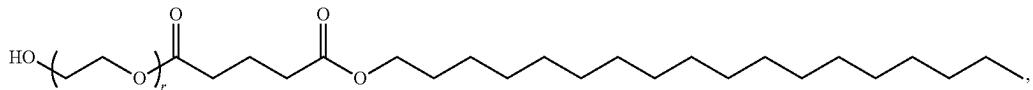
or a pharmaceutically acceptable salt thereof.

In certain embodiments, the PEG lipid of Formula (L-I) is selected from the group consisting of:

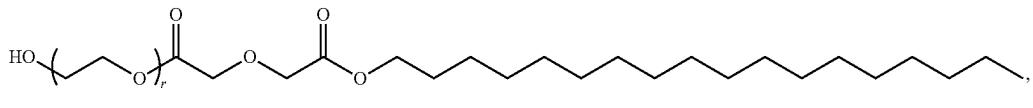
(L1)



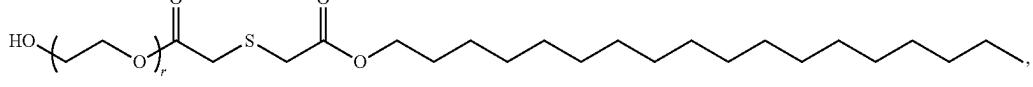
(L2)



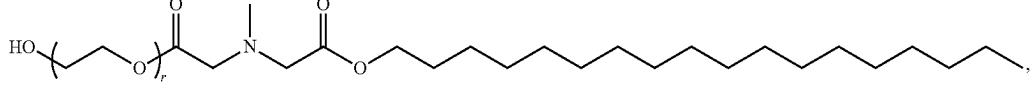
(L3)



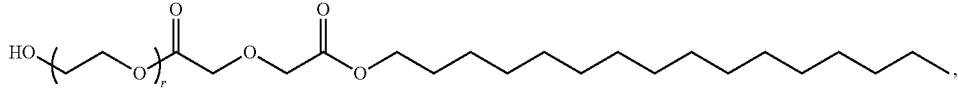
(L4)



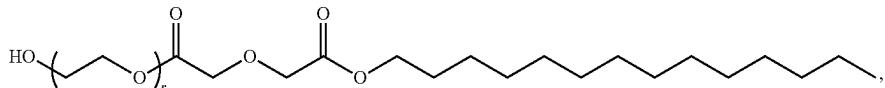
(L5)



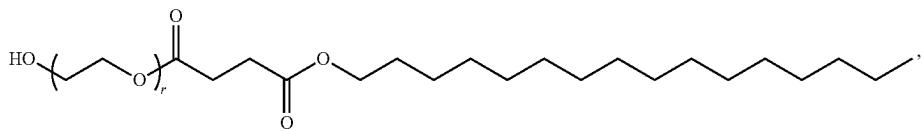
(L6)



(L7)



(L8)

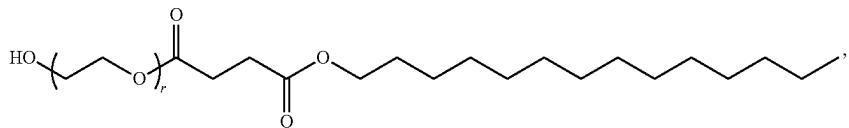


23

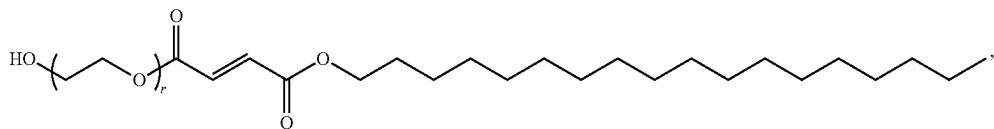
24

-continued

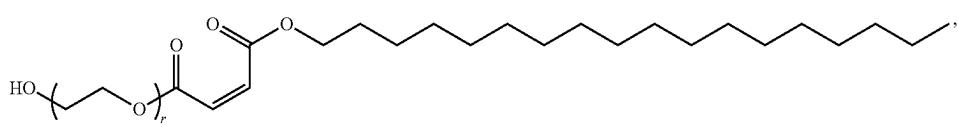
(L9)



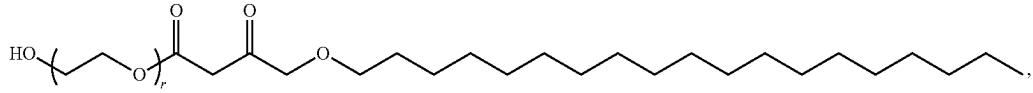
(L10)



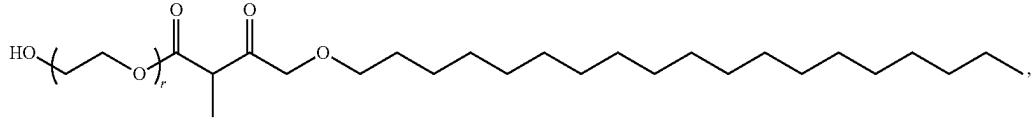
(L11)



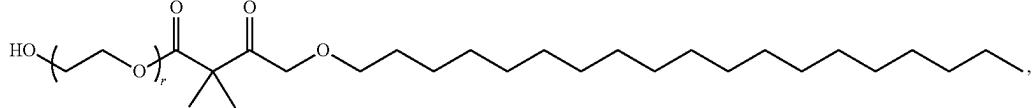
(L12)



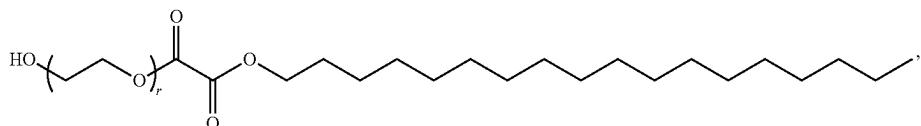
(L13)



(L14)



(L15)

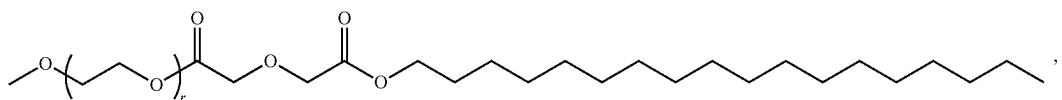


40

and pharmaceutically acceptable salts thereof.

In certain embodiments, the PEG lipid of Formula (L-I) is of the following formula:

(L40)

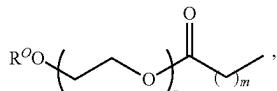


or a pharmaceutically acceptable salt thereof.

In certain embodiments, PEG lipids useful in the present invention are PEG lipids of Formula (L-II):

55

(L-II)



60

or pharmaceutically acceptable salts thereof; wherein:

R^{O} is hydrogen, optionally substituted alkyl, optionally

substituted acyl, or an oxygen protecting group;

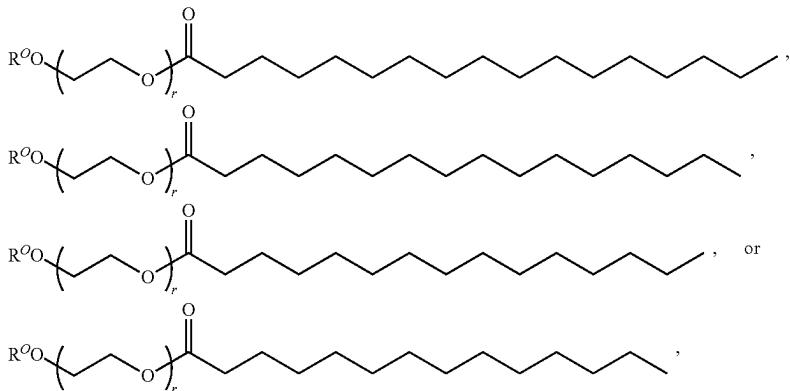
n is an integer from 2 to 100, inclusive; and

m is an integer from 5-15, inclusive, or an integer from

19-30, inclusive.

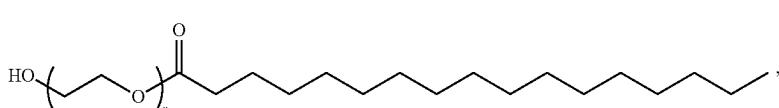
65

In certain embodiments, the PEG lipid of Formula (L-II) is of one of the following formulae:

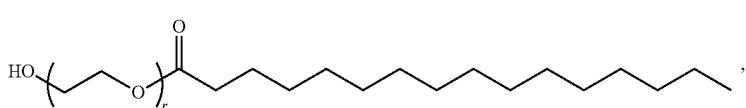


or a pharmaceutically acceptable salt thereof.

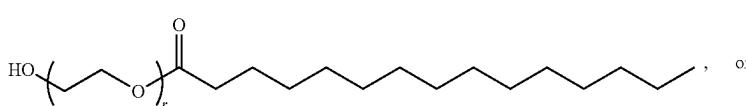
In certain embodiments, the PEG lipid of Formula (L-II) is of one of the following formulae:



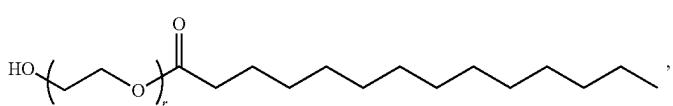
(L16)



(L17)



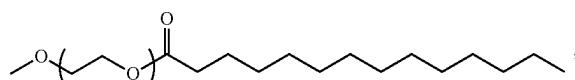
(L18)



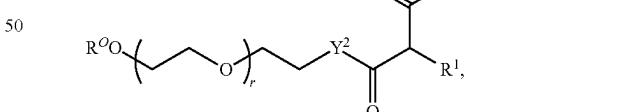
(L19)

or a pharmaceutically acceptable salt thereof.

In certain embodiments, the PEG lipid of Formula (L-II) is of the following formula:



(L39)



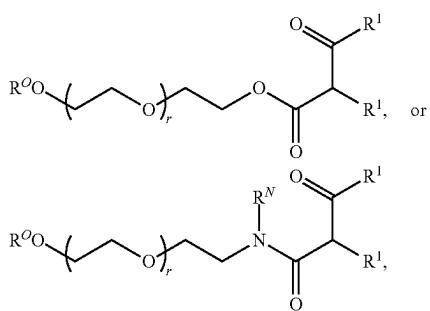
(L-III)

or pharmaceutically acceptable salts thereof, wherein:
 Y^2 is $-O-$, $-NR^N-$, or $-S-$
each instance of R^1 is independently optionally substituted C_{5-30} alkyl, optionally substituted C_{5-30} alkenyl, or optionally substituted C_{5-30} alkynyl;
 R^O is hydrogen, optionally substituted alkyl, optionally substituted acyl, or an oxygen protecting group;
 R^N is hydrogen, optionally substituted alkyl, optionally substituted acyl, or a nitrogen protecting group; and
 r is an integer from 2 to 100, inclusive.
In certain embodiments, the PEG lipid of Formula (L-III) is of one of the following formulae:

or a pharmaceutically acceptable salt thereof.

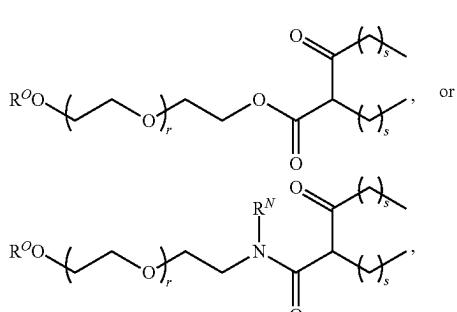
In certain embodiments, PEG lipids useful in the present invention are PEG lipids of Formula (L-III):

27



or a pharmaceutically acceptable salt thereof.

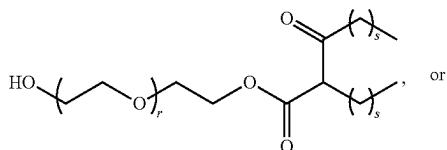
In certain embodiments, the PEG lipid of Formula (L-III) is of one of the following formulae:



28

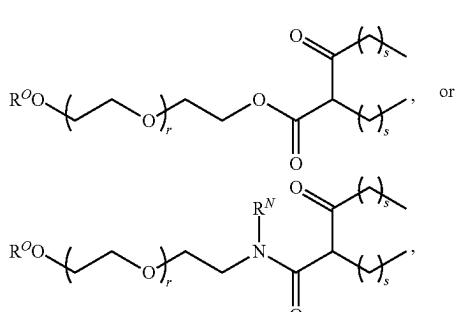
or a pharmaceutically acceptable salt thereof, wherein:
each instance of s is independently an integer from 5-25, inclusive.

5 In certain embodiments, the PEG lipid of Formula (L-III) is of one of the following formulae:

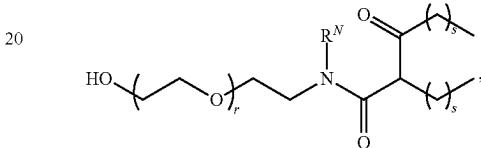


or a pharmaceutically acceptable salt thereof.

In certain embodiments, the PEG lipid of Formula (L-III) is of one of the following formulae:



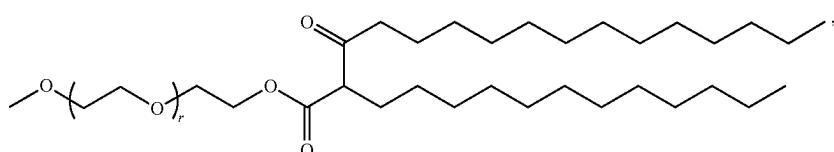
15



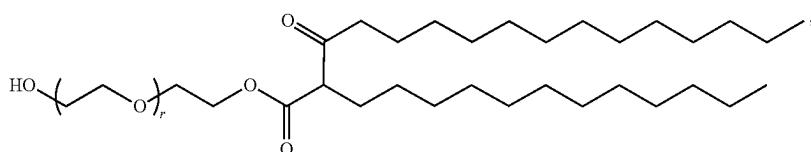
30 or a pharmaceutically acceptable salt thereof

In certain embodiments, the PEG lipid of Formula (L-III) is selected from the group consisting of:

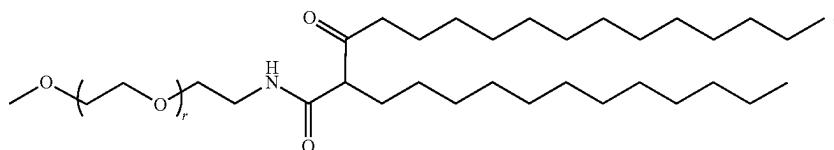
(L20)



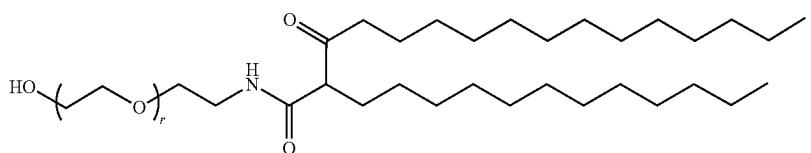
(L21)



(L22)



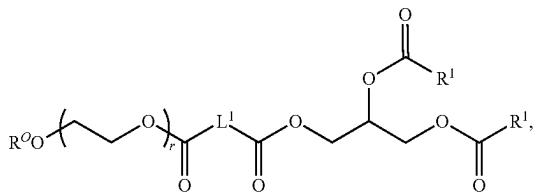
(L23)



65 and pharmaceutically acceptable salts thereof.

In certain embodiments, PEG lipids useful in the present invention are PEG lipids of Formula (L-IV):

(L-IV)



or pharmaceutically acceptable salts thereof, wherein:

L¹ is a bond, optionally substituted C₁₋₃ alkylene, optionally substituted C₁₋₃ heteroalkylene, optionally substituted C₂₋₃ alkenylene, optionally substituted C₂₋₃ alkynylene;

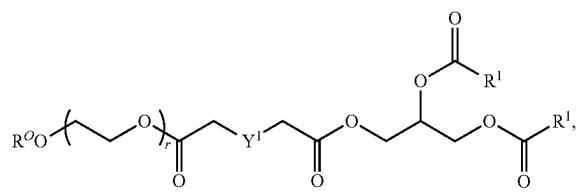
each instance of R¹ is independently optionally substituted C₅₋₃₀ alkyl, optionally substituted C₃₋₃₀ alkenyl, or optionally substituted C₅₋₃₀ alkynyl;

R^O is hydrogen, optionally substituted alkyl, optionally substituted acyl, or an oxygen protecting group;

R^N is hydrogen, optionally substituted alkyl, optionally substituted acyl, or a nitrogen protecting group;

r is an integer from 2 to 100, inclusive.

In certain embodiments, the PEG lipid of Formula (L-IV) is of the formula:

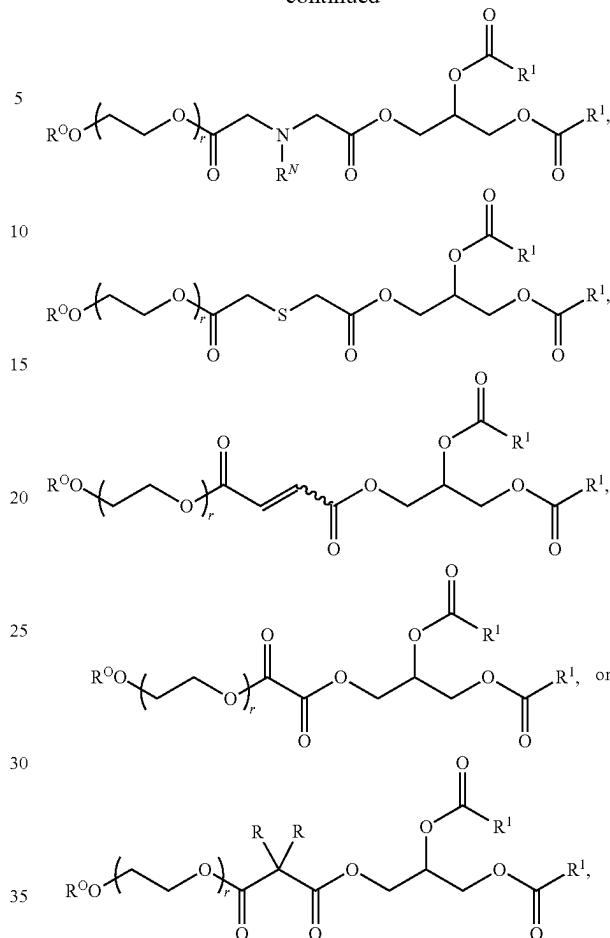


or a pharmaceutically acceptable salt thereof, wherein:

Y¹ is a bond, -CR₂, -O-, -NR^N, or -S-; and each instance of R is independently hydrogen, halogen, or optionally substituted alkyl.

In certain embodiments, the PEG lipid of Formula (L-IV) is of one of the following formulae:

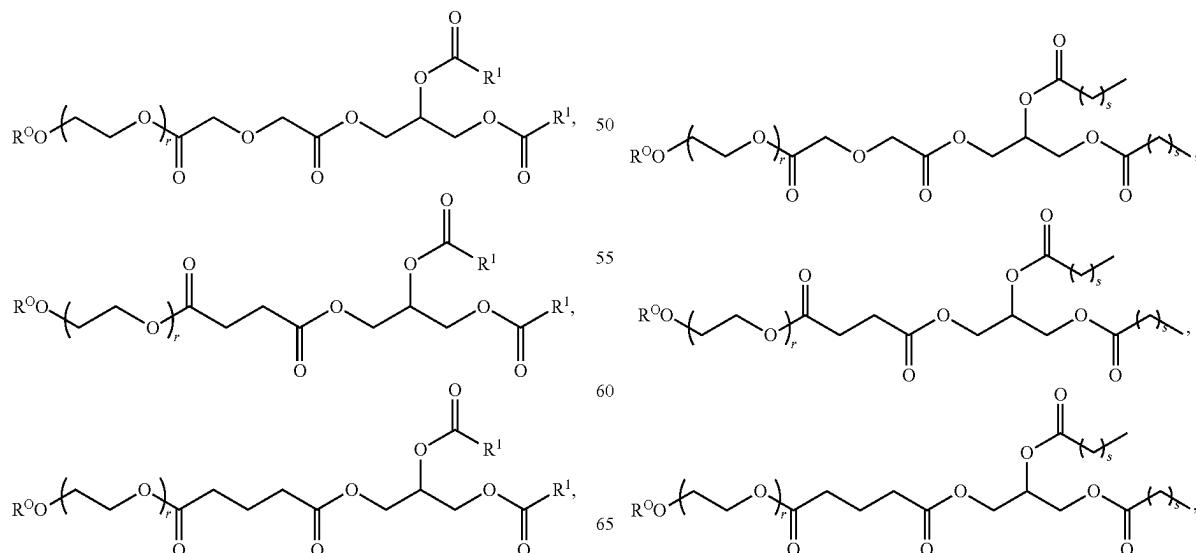
-continued



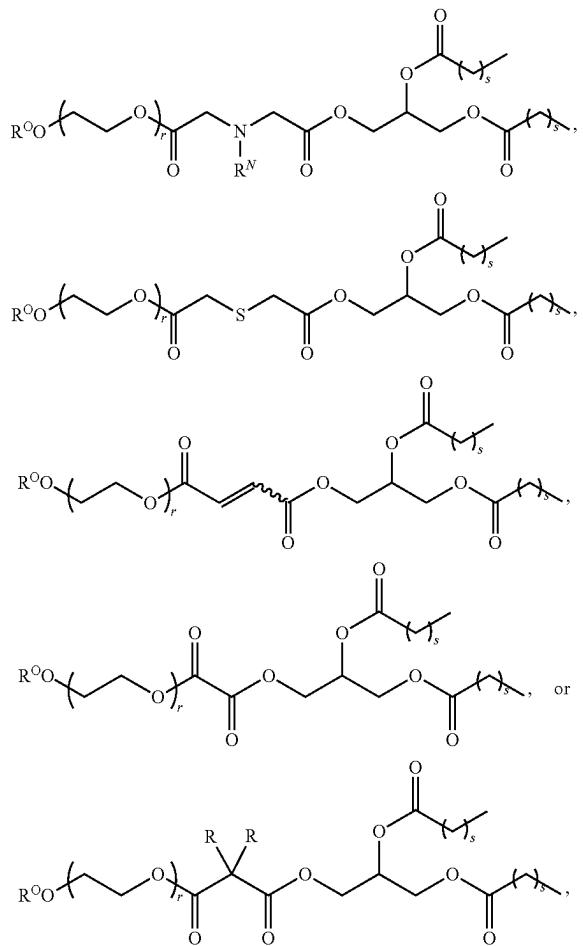
or a pharmaceutically acceptable salt thereof, wherein:

each instance of R is independently hydrogen, halogen, or optionally substituted alkyl.

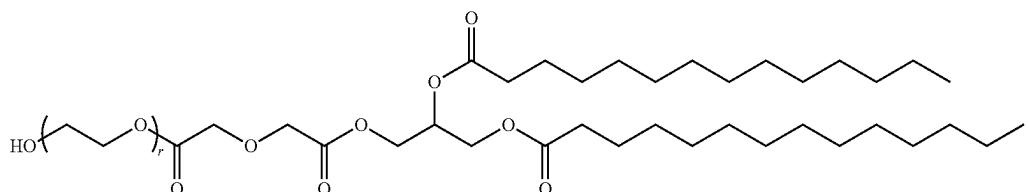
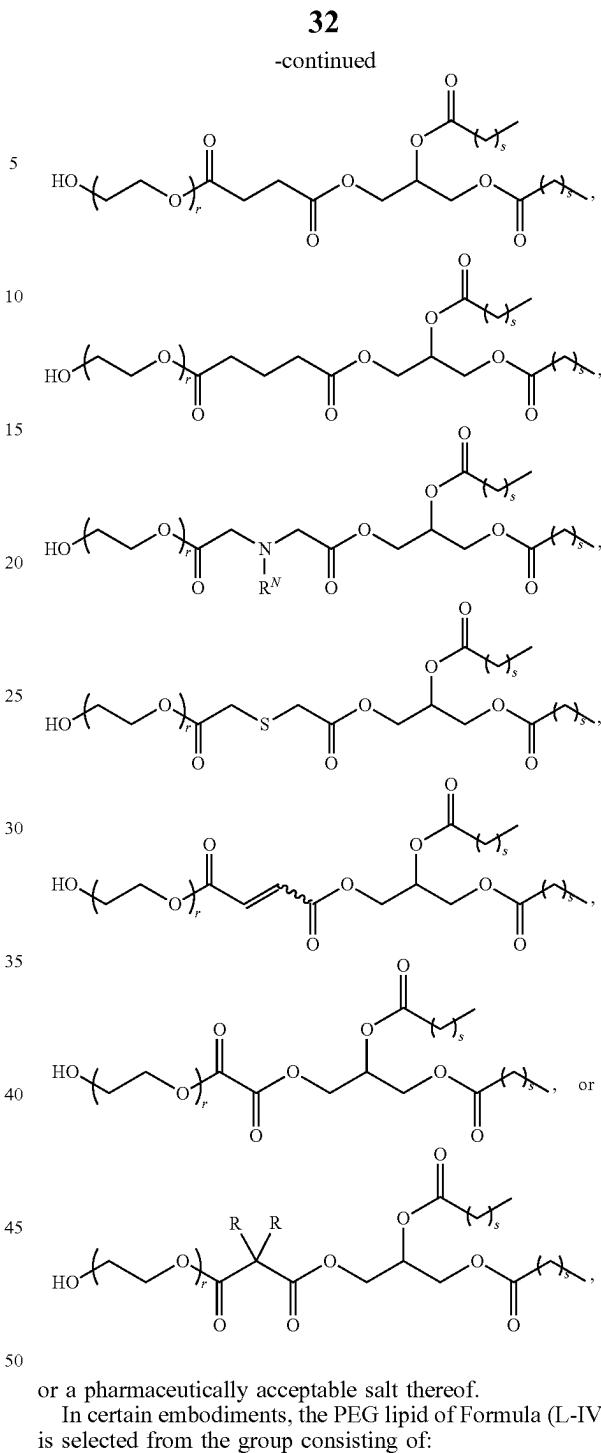
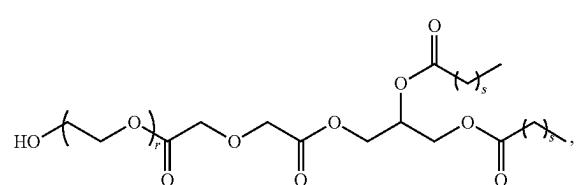
In certain embodiments, the PEG lipid of Formula (L-IV) is of one of the following formulae:



31
-continued

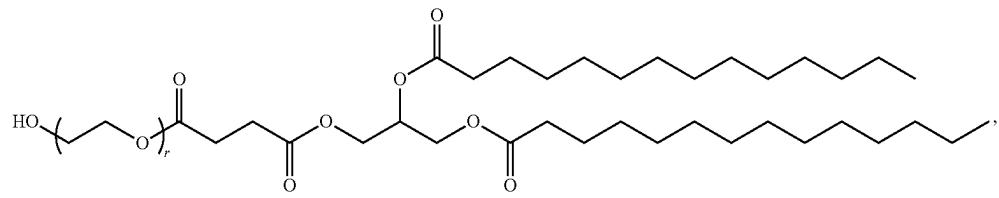


or a pharmaceutically acceptable salt thereof, wherein:
each s is independently an integer from 5-25, inclusive.
In certain embodiments, the PEG lipid of Formula (L-IV)
is of one of the following formulae:

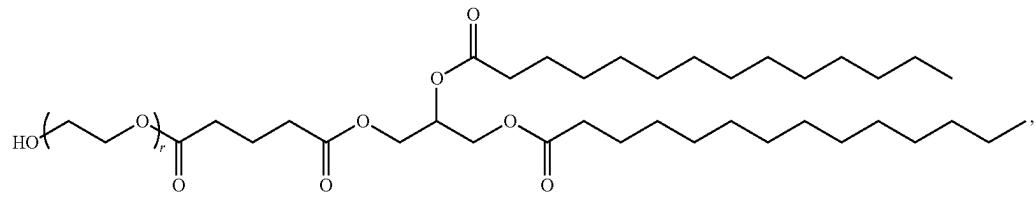


-continued

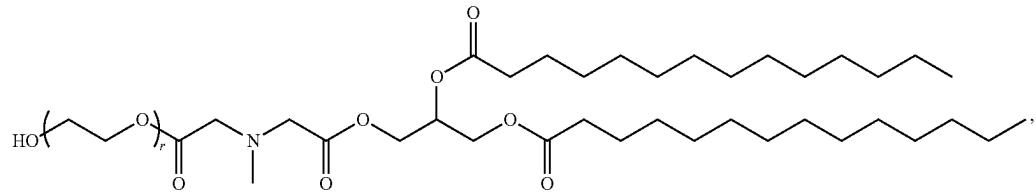
(L25)



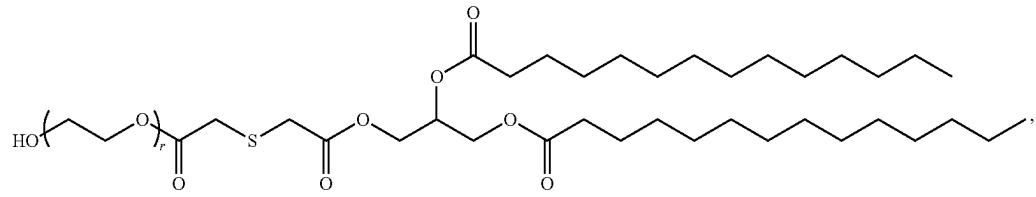
(L26)



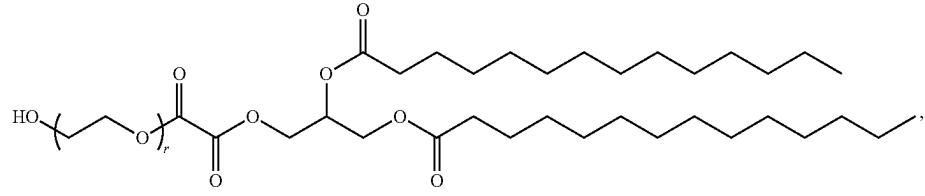
(L27)



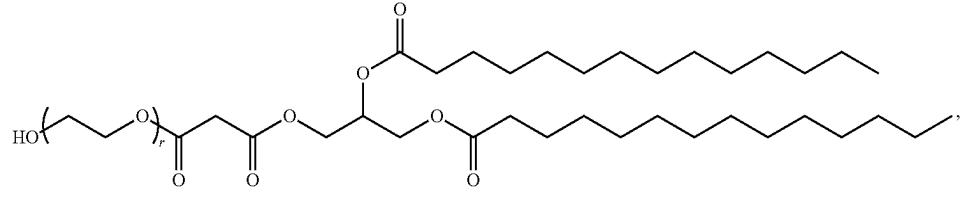
(L28)



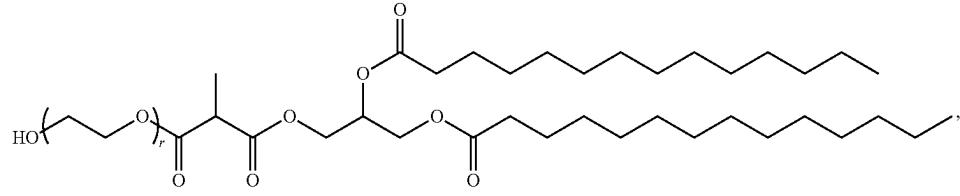
(L29)



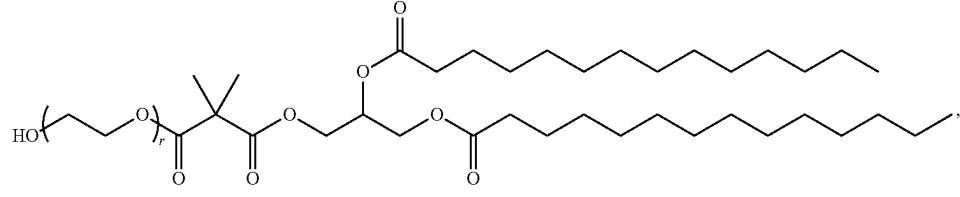
(L30)



(L31)



(L32)

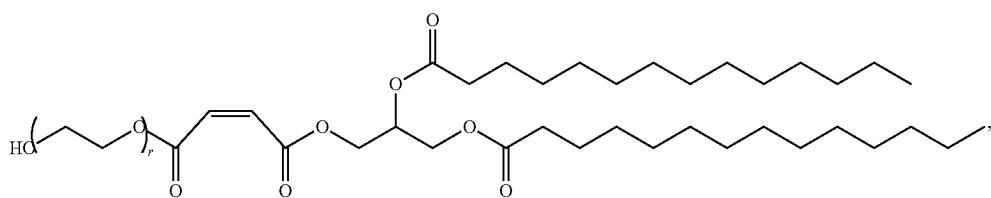
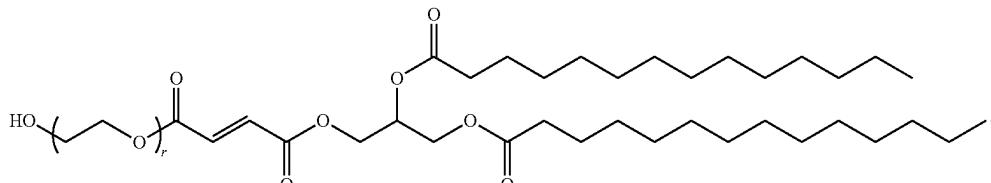


35

-continued

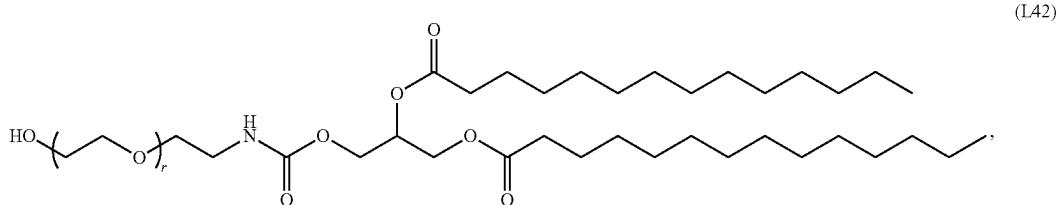
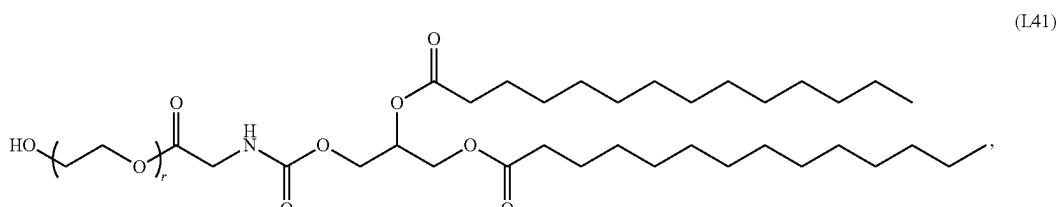
36

(L33)



and pharmaceutically acceptable salts thereof. 20

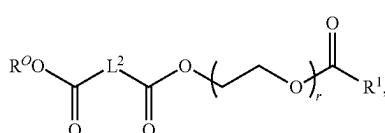
In certain embodiments, the PEG lipid is selected from the group consisting of:



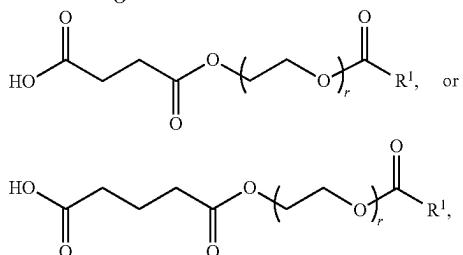
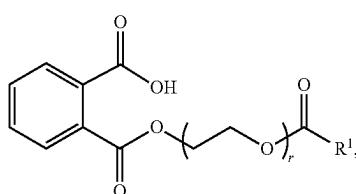
and pharmaceutically acceptable salts thereof.

In certain embodiments, the PEG lipid is of Formula (L-V):

45



50



and pharmaceutically acceptable salts thereof, wherein:

L^2 is a bond, optionally substituted C_{1-6} alkylene, optionally substituted C_{1-6} heteroalkylene, optionally substituted C_{2-6} alkenylene, optionally substituted C_{2-6} alkynylene, optionally substituted arylene, optionally substituted heteroarylene, optionally substituted heterocyclylene, or optionally substituted carbocyclylene, or a combination thereof;

each instance of R^1 is independently optionally substituted C_{5-30} alkyl, optionally substituted C_{5-30} alkenyl, or optionally substituted C_{5-30} alkynyl;

R^O is hydrogen, optionally substituted alkyl, optionally

substituted acyl, or an oxygen protecting group; and

r is an integer from 2 to 100, inclusive.

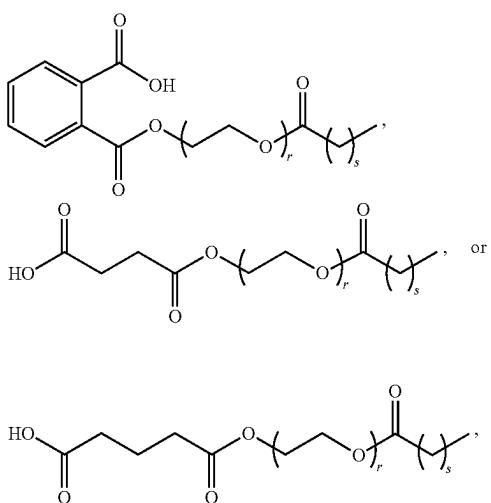
In certain embodiments, the PEG lipid of Formula (L-V) is of one of the following formulae:

45

L^2 is a bond, optionally substituted C_{1-6} alkylene, optionally substituted C_{1-6} heteroalkylene, optionally substituted C_{2-6} alkenylene, optionally substituted C_{2-6} alkynylene, optionally substituted arylene, optionally substituted heteroarylene, optionally substituted heterocyclylene, or optionally substituted carbocyclylene, or a combination thereof;

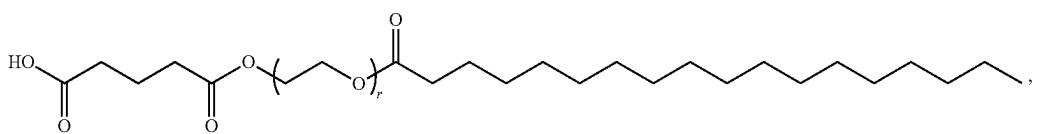
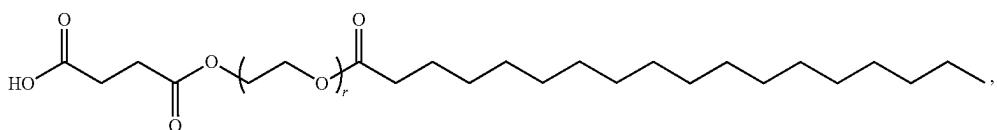
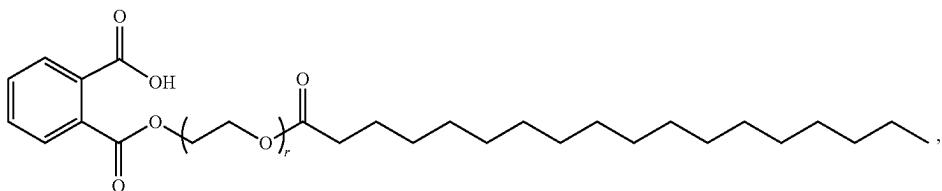
R^1 is a pharmaceutically acceptable salt thereof.

In certain embodiments, the PEG lipid of Formula (L-V) is of one of the following formulae:



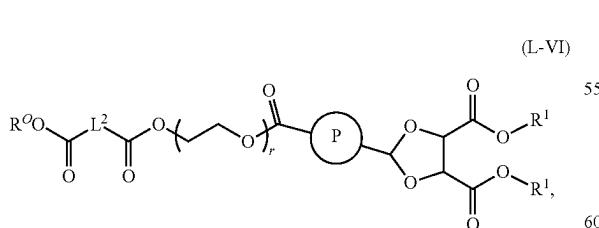
or a pharmaceutically acceptable salt thereof.

In certain embodiments, the PEG lipid of Formula (L-V) is selected from the group consisting of:



and pharmaceutically acceptable salts thereof.

In certain embodiments, the PEG lipid is of Formula (L-VI):



and pharmaceutically acceptable salts thereof, wherein:

L^2 is a bond, optionally substituted C_{1-6} alkylene, optionally substituted C_{1-6} heteroalkylene, optionally substituted C_{2-6} alkenylene, optionally substituted C_{2-6} alkynylene, or optionally substituted arylene, optionally

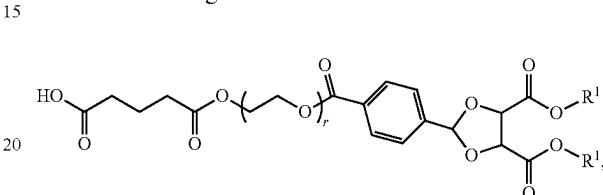
substituted heteroarylene, optionally substituted heterocyclyene, or optionally substituted carbocyclyene, or combinations thereof;

Ring P is optionally substituted aryl, optionally substituted heteroaryl, optionally substituted heterocycl, or optionally substituted carbocycl;

each instance of R^1 is independently optionally substituted C_{5-30} alkyl, optionally substituted C_{3-30} alkenyl, or optionally substituted C_{5-30} alkynyl;

R^2 is hydrogen, optionally substituted alkyl, optionally substituted acyl, or an oxygen protecting group; and r is an integer from 2 to 100, inclusive.

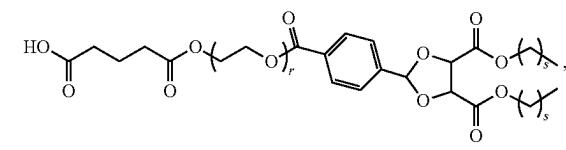
In certain embodiments, the PEG lipid of Formula (L-VI) is of the following formula:



or a pharmaceutically acceptable salt thereof.

(L38)

In certain embodiments, the PEG lipid of Formula (L-VI) is of the following formula:

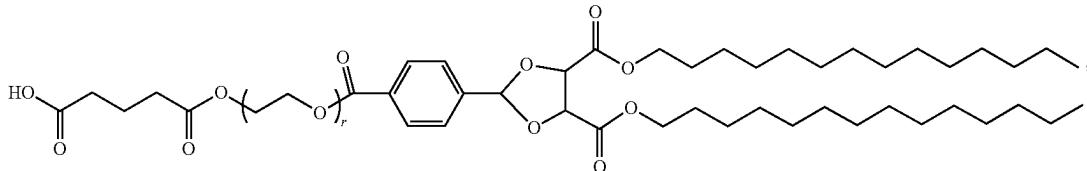


or a pharmaceutically acceptable salt thereof, wherein:

each s is independently an integer from 5-25, inclusive.

In certain embodiments, the PEG lipid of Formula (L-VI) is of the following formula:

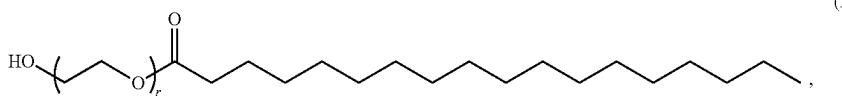
(L38)



or a pharmaceutically acceptable salt thereof.

High Purity PEG Lipids of Formula (I)

In certain embodiments, the plurality of PEG lipids is a plurality of compounds of Formula (I), or salts thereof. Provided herein are pluralities of compounds of Formula (I):



or salts thereof; wherein r is independently an integer from 35-55, inclusive; and a plurality of compounds of Formula (I) has a purity of 87% or greater with respect to compounds of Formula (I).

As defined herein, r is an integer from 35-55, inclusive. In certain embodiments, r is independently an integer from 36-54, inclusive. In certain embodiments, r is independently an integer from 37-53, inclusive. In certain embodiments, r is independently an integer from 38-52, inclusive. In certain embodiments, r is independently an integer from 39-51, inclusive. In certain embodiments, r is independently an integer from 40, inclusive. In certain embodiments, r is independently an integer from 40-50, inclusive. In certain embodiments, r is independently an integer from 41-49, inclusive. In certain embodiments, r is independently an integer from 42-48, inclusive. In certain embodiments, r is independently an integer from 43-57, inclusive. In certain embodiments, r is independently an integer from 44-46, inclusive. In certain embodiments, r is 45.

In certain embodiments, r is independently 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55. In certain embodiments, r is independently 35. In certain embodiments, r is independently 36. In certain embodiments, r is independently 37. In certain embodiments, r is independently 38. In certain embodiments, r is independently 39. In certain embodiments, r is independently 40. In certain embodiments, r is independently 41. In certain embodiments, r is independently 42. In certain embodiments, r is independently 43. In certain embodiments, r is independently 44. In certain embodiments, r is independently 45. In certain embodiments, r is independently 46. In certain embodiments, r is independently 47. In certain embodiments, r is independently 48. In certain embodiments, r is independently 49. In certain embodiments, r is independently 50. In certain embodiments, r is independently 51. In certain embodiments, r is independently 52. In certain embodiments, r is independently 53. In certain embodiments, r is independently 54. In certain embodiments, r is independently 55.

It is to be understood that a "plurality" of compounds of Formula (I) may comprise a mixture of compounds of Formula (I) having different values for r (i.e., having different length PEG chains). In such an instance, the recommended purity

(87% or greater, with several embodiments provided below) refers to all compounds of Formula (I) in the mixture taken together. For example, a plurality of compounds of Formula (I) with a purity of approximately 98% comprises 98% (mol %) compounds of Formula (I), with each compound of

25 Formula (I) in the mixture having, independently, a value for r from 35-55, inclusive. In certain embodiments, greater than 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% of the compounds have a value of r from 40-50, inclusive. In certain embodiments, greater than 20%, 30%, 30, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% of the compounds have a value of r from 42-48, inclusive. In certain embodiments, greater than 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% of the compounds have a value of r from 43-46, inclusive. In certain embodiments, greater than 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% of the compounds have r=45.

As described herein, the plurality of compounds of Formula (I), or salts thereof, has a purity greater than 87% with respect to the compounds of Formula (I), or the salts thereof. 40 In certain embodiments, the purity is greater than 90%. In certain embodiments, the purity is greater than 91%. In certain embodiments, the purity is greater than 92%. In certain embodiments, the purity is greater than 93%. In certain embodiments, the purity is greater than 94%. In certain embodiments, the purity is greater than 95%. In certain embodiments, the purity is greater than 96%. In certain embodiments, the purity is greater than 97%. In certain embodiments, the purity is greater than 98%. In certain embodiments, the purity is greater than 98.5%. In certain embodiments, the purity is greater than 99%. In certain embodiments, the purity is greater than 99.1%. In certain embodiments, the purity is greater than 99.2%. In certain embodiments, the purity is greater than 99.3%. In certain embodiments, the purity is greater than 99.4%. In 55 certain embodiments, the purity is greater than 99.5%. In certain embodiments, the purity is greater than 99.6%. In certain embodiments, the purity is greater than 99.7%. In certain embodiments, the purity is greater than 99.8%. In certain embodiments, the purity is greater than 99.9%. In certain embodiments, the purity is 100%.

In certain embodiments, the plurality of compounds of Formula (I), or salts thereof, has a purity from 87-100%, inclusive, with respect to the compounds of Formula (I) or salts thereof. In certain embodiments, the purity is from 87-99.9%, inclusive.

In certain embodiments, the purity is from 87-90%, inclusive. In certain embodiments, the purity is from

41

90-95%, inclusive. In certain embodiments, the purity is from 95-100%, inclusive. In certain embodiments, the purity is from 95-99.9%, inclusive.

In certain embodiments, the purity is from 90-100%, inclusive. In certain embodiments, the purity is from 90-99.9%, inclusive. In certain embodiments, the purity is from 91-99.9%, inclusive. In certain embodiments, the purity is from 92-99.9%, inclusive. In certain embodiments, the purity is from 93-99.9%, inclusive. In certain embodiments, the purity is from 94-99.9%, inclusive. In certain embodiments, the purity is from 95-99.9%, inclusive. In certain embodiments, the purity is from 96-99.9%, inclusive. In certain embodiments, the purity is from 97-99.9%, inclusive. In certain embodiments, the purity is from 98-99.9%, inclusive. In certain embodiments, the purity is from 99-99.9%, inclusive.

In certain embodiments the plurality of compounds of Formula (I), or salts thereof, has a purity of approximately 87% with respect to the compounds of Formula (I) or the salts thereof. In certain embodiments, the purity is approximately 87.5%. In certain embodiments, the purity is approximately 88%. In certain embodiments, the purity is approximately 88.5%. In certain embodiments, the purity is approximately 89%. In certain embodiments, the purity is approximately 89.5%. In certain embodiments, the purity is approximately 90%. In certain embodiments, the purity is approximately 90.5%. In certain embodiments, the purity is approximately 91%. In certain embodiments, the purity is approximately 91.5%. In certain embodiments, the purity is approximately 92%. In certain embodiments, the purity is approximately 92.5%. In certain embodiments, the purity is approximately 93%. In certain embodiments, the purity is approximately 93.5%. In certain embodiments, the purity is approximately 94%. In certain embodiments, the purity is approximately 94.5%. In certain embodiments, the purity is approximately 95%. In certain embodiments, the purity is approximately 95.5%. In certain embodiments, the purity is approximately 96%. In certain embodiments, the purity is approximately 96.6%. In certain embodiments, the purity is approximately 97%. In certain embodiments, the purity is approximately 97.7%. In certain embodiments, the purity is approximately 98%. In certain embodiments, the purity is approximately 98.5%. In certain embodiments, the purity is approximately 99%. In certain embodiments, the purity is approximately 99.5%. In certain embodiments, the purity is approximately 99.9%. In certain embodiments, the purity is approximately 100%. In certain embodiments, the purity is approximately 98.1%, 98.2%, 98.3%, 98.4%, 98.5%, 98.6%,

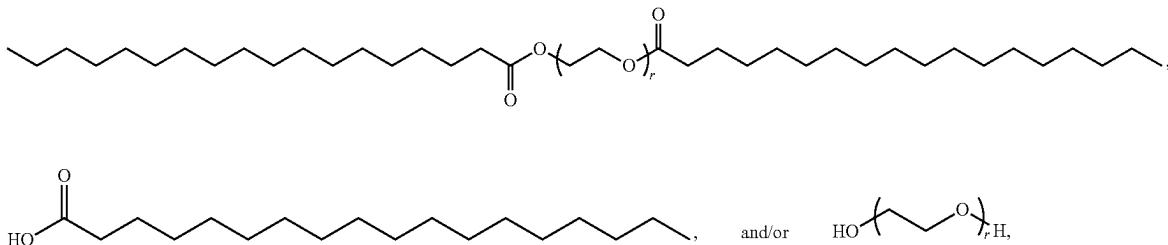
42

In certain embodiment, the plurality of compounds consists of compounds of Formula (I), or salts thereof. In certain embodiments, the plurality of compounds consists essentially of compounds of Formula (I), or salts thereof.

In certain embodiments, the plurality of compounds of Formula (I), or salts thereof, is substantially free of other compounds. In certain embodiments, the plurality of compounds is substantially free of impurities. The term "impurities" refers to compounds other than the compounds of Formula (I) or salt thereof. In certain embodiments, the plurality of compounds comprises less than 13% of total impurities. In certain embodiments, the plurality of compounds comprises less than 12% of total impurities. In certain embodiments, the plurality of compounds comprises less than 11% of total impurities. In certain embodiments, the plurality of compounds comprises less than 10% of total impurities. In certain embodiments, the plurality of compounds comprises less than 9% of total impurities. In certain embodiments, the plurality of compounds comprises less than 8% of total impurities. In certain embodiments, the plurality of compounds comprises less than 7% of total impurities. In certain embodiments, the plurality of compounds comprises less than 6% of total impurities. In certain embodiments, the plurality of compounds comprises less than 5% of total impurities. In certain embodiments, the plurality of compounds comprises less than 4% of total impurities. In certain embodiments, the plurality of compounds comprises less than 3% of total impurities. In certain embodiments, the plurality of compounds comprises less than 2% of total impurities. In certain embodiments, the plurality of compounds comprises less than 1% of total impurities.

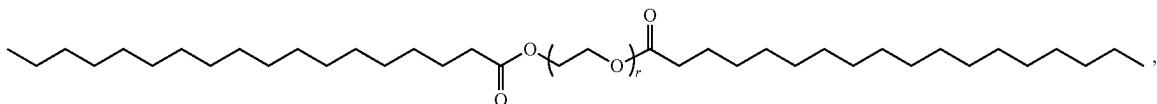
In certain embodiments, the PEG lipids in the plurality of compounds of Formula (I) are the same compound. In certain embodiment, the plurality of PEG lipids comprises a mixture of compounds of Formula (I). A plurality of compounds of Formula (I) provided herein may comprise other components such as solvents, carriers, excipients, etc., and can therefore be in the form of a solution or composition. When the compounds of Formula (I) are part of a solution or composition, it is to be understood that the proscribed/recommended purity (e.g., of 87% or greater) is with respect to the compounds of Formula (I) component of the solution or composition.

In certain embodiments, the impurities include one or more fatty acids. In certain embodiments, the impurities include one or more PEG polymers. In certain embodiments, the impurities include one or more fatty acids and/or one or more PEG polymers. In certain embodiments, the impurities are one or more of the following:

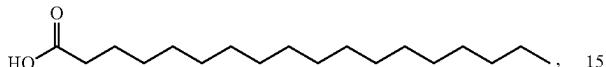


98.7%, 98.8%, or 98.9%. In certain embodiments, the purity is approximately 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%.

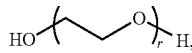
or salts thereof, wherein r is independently an integer from 35-55, inclusive. In certain embodiments, the impurities include compounds of the formula:



or salts thereof, wherein r is independently an integer from 35-55, inclusive. In certain embodiments, impurities include the compound:

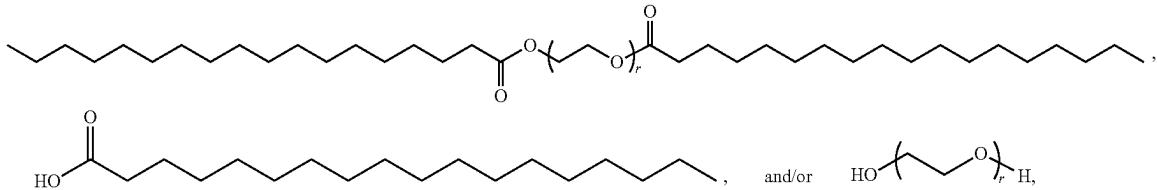


or salts thereof. In certain embodiments, the impurities include polymers of the formula:

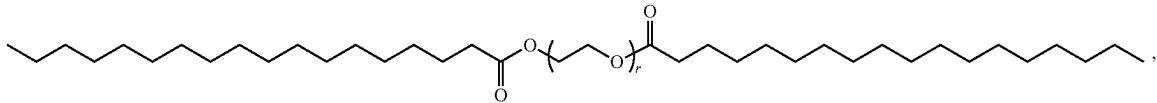


or salts thereof, wherein r is independently an integer from 35-55, inclusive.

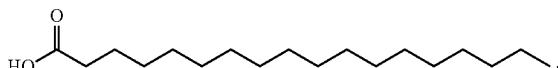
In certain embodiments, the plurality of compounds is substantially free of PEG. In certain embodiments, the plurality of compounds is substantially free of fatty acids. In certain embodiments, the plurality of compounds of Formula (I), or salts thereof, is substantially free of one or more of the following:



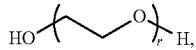
or salts thereof, wherein r is independently an integer from 35-55, inclusive. In certain embodiments, the plurality of compounds of Formula (I), or salts thereof, is substantially free of compounds of the formula:



or salts thereof, wherein r is independently an integer from 35-55, inclusive. In certain embodiments, the plurality of compounds of Formula (I), or salts thereof, is substantially free of the compound:



or a salt thereof. In certain embodiments, the plurality of compounds of Formula (I), or salts thereof, is substantially free of polymers of the formula:



or salts thereof, wherein r is independently an integer from 35-55, inclusive. "Substantially free," when used herein in regard to an impurity, refers to a maximum of 0-5% of impurity present. In certain embodiments, 0-2% of impurity may be present. In certain embodiments, 0-1% of impurity may be present.

The polydispersity index (PDI) of PEG lipids provided herein (e.g., compounds of Formula (I)) can be measured. "Polydispersity index" refers to the measure of heterogeneity of sizes of molecules in a mixture, and is also referred to as the "heterogeneity index" or "dispersity." In other terms, it is the measure of the distribution of molecular mass in a given polymer sample. PDI is calculated based on the following formula: $PDI = M_w/M_n$, wherein M_w is the weight average molecular weight, and M_n is the number average molecular weight. As the polymer chains (e.g., PEG chains)

in a mixture of polymers approach uniform chain length, PDI will approach a value of 1. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.01, 1.02, 1.03, 1.04, 1.05, 1.06, 1.07, 1.08, 1.09, 1.1, or less. In

certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, or less. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.0 to 1.10, inclusive. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.02 to 1.10, inclusive. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.0 to 1.04, inclusive. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.02 to 1.04, inclusive. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of 1.10 or less. In certain embodiments, a plurality of PEG lipids provided herein has

a PDI of 1.04 or less. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of approximately 1.04. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of approximately 1.03. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of approximately 1.02. In certain embodiments, a plurality of PEG lipids provided herein has a PDI of approximately 1.01.

Lipid Nanoparticles (LNPs) Comprising High-Purity PEG Lipids

Provided herein are lipid nanoparticles (LNPs) comprising the high-purity PEG lipids described herein. In other words, provided herein are lipid nanoparticles (LNPs) comprising a plurality of PEG lipids, wherein the plurality of PEG lipids has a purity of 87% or greater with respect to the PEG lipid component of the LNP. Any PEG lipids described herein may constitute the high-purity PEG lipid component of the LNP provided herein.

When "PEG lipids" or "high-purity PEG lipids" are referred to as components in an LNP formulation, it is implied that the PEG lipid mixture has the proscribed/recommended purity of 87% or greater with respect to the PEG lipids. Several other embodiments (i.e., recommended purities) are provided in the section above. In addition to the PEG lipids provided herein, the LNP may comprise one or more additional lipid components.

Provided herein are lipid nanoparticles (LNPs) comprising a plurality of PEG lipids provided herein (e.g., a plurality of compounds of Formula (I) with a purity of 87% or greater as provided herein). In certain embodiments, the PEG lipids are present in the LNP in a molar ratio of 0.15-15% with respect to other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of 0.15-5% with respect to other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of 1-2% with respect to other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of approximately 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, or 2% with respect to other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of approximately 1.5% with respect to other lipids.

Examples of PEG lipids are provided herein, any of which can be used in the recommended purity of 87% or greater. In certain embodiments, the PEG lipids are a plurality of compounds of Formula (I) as described herein. In certain embodiments, the plurality of compounds of Formula (I) is present in the LNP in a molar ratio of 0.15-15% with respect to other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 0.15-5% with respect to other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 1-5% with respect to other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 0.15-2% with respect to other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 1-2% with respect to other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of approximately 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, or 2% with respect to other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of approximately 1.5% with respect to other lipids.

LNPs provided herein may comprise any lipid(s) in addition to the high-purity PEG lipid component. As generally defined herein, the term "lipid" refers to a small molecule that has hydrophobic or amphiphilic properties.

5 Lipids may be naturally occurring or synthetic. Examples of classes of lipids include, but are not limited to, fats, waxes, sterol-containing metabolites, vitamins, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides, and prenol lipids. In some instances, the amphiphilic properties of some lipids leads them to form liposomes, vesicles, or membranes in aqueous media.

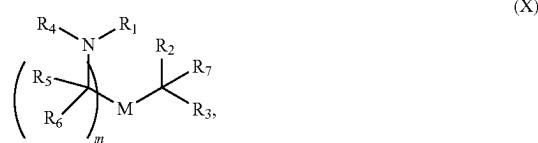
In certain embodiments, for example, a lipid nanoparticle may comprise a PEG lipid, an ionizable amino lipid, a helper lipid, and a structural lipid.

Lipid Nanoparticles (LNPs) Comprising High-Purity PEG Lipids and Ionizable Amino Lipids

In certain embodiments, the LNP comprises an ionizable amino lipid (i.e., cationic lipid).

Provided herein are LNPs comprising a high-purity PEG lipid component described herein (i.e., with the proscribed/recommended purity of 87% or greater with respect to the PEG lipid component), and an ionizable amino lipid. In certain embodiments, the LNP comprises a high-purity PEG lipid component described herein, and a compound of Formula (X). In certain embodiments, the LNP comprises a plurality of compounds of Formula (I) described herein (i.e., with the proscribed/recommended purity of 87% or greater with respect to the PEG lipid component) and a compound of Formula (X).

A compound of Formula (X) has the following formula:



or a salt or isomer thereof, wherein:

R₁ is selected from the group consisting of C₅₋₃₀ alkyl, C₅₋₂₀ alkenyl, —R*YR", —YR", and —R"MR'";

R₂ and R₃ are independently selected from the group consisting of H, C₁₋₁₄ alkyl, C₂₋₁₄ alkenyl, —R*YR", —YR", and —R"OR", or R₂ and R₃, together with the atom to which they are attached, form a heterocycle or carbocycle;

R₄ is selected from the group consisting of a C₃₋₆ carbocycle, —(CH₂)_nQ, —(CH₂)_nCHQR, —CHQR, —CQ(R)₂, and unsubstituted C₁₋₆ alkyl, where Q is selected from a carbocycle, heterocycle, —OR, —O(CH₂)_nN(R)₂, —C(O)OR, —OC(O)R, —CX₃, —CX₂H, —CXH₂, —CN, —N(R)₂, —C(O)N(R)₂, —N(R)C(S)N(R)₂, —N(R)S(O)₂R, —N(R)C(O)N(R)₂, —N(R)C(S)N(R)₂, —N(R)R₈, —O(CH₂)_nOR, —N(R)C(=NR₉)N(R)₂, —N(R)C(=CHR₉)N(R)₂, —OC(O)N(R)₂, —N(R)C(O)OR, —N(OR)C(O)R, —N(OR)S(O)₂R, —N(OR)C(O)OR, —N(OR)C(O)N(R)₂, —N(OR)C(S)N(R)₂, —N(OR)C(=NR₉)N(R)₂, —N(OR)C(=CHR₉)N(R)₂, —C(=NR₉)N(R)₂, —C(=NR₉)R, —C(O)N(R)OR, and —C(R)N(R)₂C(O)OR, and each n is independently selected from 1, 2, 3, 4, and 5;

each R₅ is independently selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H;

each R₆ is independently selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H;

M and M' are independently selected from $-\text{C}(\text{O})\text{O}-$, $-\text{OC}(\text{O})-$, $-\text{C}(\text{O})\text{N}(\text{R}')-$, $-\text{N}(\text{R}')\text{C}(\text{O})-$, $-\text{C}(\text{O})-$, $-\text{C}(\text{S})-$, $-\text{C}(\text{S})\text{S}-$, $-\text{SC}(\text{S})-$, $-\text{CH}(\text{OH})-$, $-\text{P}(\text{O})(\text{OR}')\text{O}-$, $-\text{S}(\text{O})_2-$, $-\text{S}-\text{S}-$, an aryl group, and a heteroaryl group;

R₇ is selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H;

R₈ is selected from the group consisting of C₃₋₆ carbocycle and heterocycle;

R₉ is selected from the group consisting of H, CN, NO₂, C₁₋₆ alkyl, $-\text{OR}$, $-\text{S}(\text{O})_2\text{R}$, $-\text{S}(\text{O})_2\text{N}(\text{R})_2$, C₂₋₆ alkenyl, C₃₋₆ carbocycle and heterocycle;

each R is independently selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H;

each R' is independently selected from the group consisting of C₁₋₁₈alkyl, C₂₋₁₈ alkenyl, $-\text{R}^*\text{YR}''$, $-\text{YR}''$, and H;

each R'' is independently selected from the group consisting of C₃₋₁₄ alkyl and C₃₋₁₄ alkenyl;

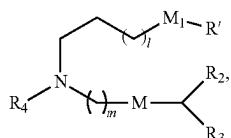
each R* is independently selected from the group consisting of C₁₋₁₂ alkyl and C₂₋₁₂ alkenyl;

each Y is independently a C₃₋₆ carbocycle;

each X is independently selected from the group consisting of F, Cl, Br, and I; and

m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13.

In some embodiments, a compound of Formula (X) is of Formula (XA):

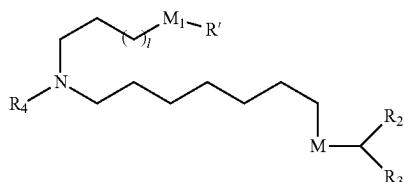


(XA)

30
35

or a salt or isomer thereof, wherein 1 is selected from 1, 2, 3, 4, and 5; m is selected from 5, 6, 7, 8, and 9; M₁ is a bond or M'; R₄ is unsubstituted C₁₋₃ alkyl, or $-(\text{CH}_2)_n\text{Q}$, in which Q is OH, $-\text{NHC}(\text{S})\text{N}(\text{R})_2$, $-\text{NHC}(\text{O})\text{N}(\text{R})_2$, $-\text{N}(\text{R})\text{C}(\text{O})\text{O}\text{R}$, $-\text{N}(\text{R})\text{S}(\text{O})_2\text{R}$, $-\text{N}(\text{R})\text{R}_8$, $-\text{NHC}(=\text{NR}_9)\text{N}(\text{R})_2$, $-\text{NHC}(=\text{CHR}_9)\text{N}(\text{R})_2$, $-\text{OC}(\text{O})\text{N}(\text{R})_2$, $-\text{N}(\text{R})\text{C}(\text{O})\text{OR}$, heteroaryl or heterocycloalkyl; M and M' are independently selected from $-\text{C}(\text{O})\text{O}-$, $-\text{OC}(\text{O})-$, $-\text{C}(\text{O})\text{N}(\text{R}')-$, $-\text{P}(\text{O})(\text{OR}')\text{O}-$, $-\text{S}-\text{S}-$, an aryl group, and a heteroaryl group; and R₂ and R₃ are independently selected from the group consisting of H, C₁₋₁₄ alkyl, and C₂₋₁₄ alkenyl.

In certain embodiments, the compound of Formula (X) is of Formula (XI):



(XI)

50
55
60

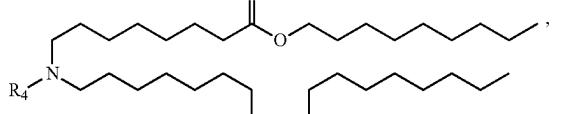
or a salt or isomer thereof, wherein 1 is selected from 1, 2, 3, 4, and 5; M₁ is a bond or M'; R₄ is unsubstituted C₁₋₃ alkyl, or $-(\text{CH}_2)_n\text{Q}$, in which n is 2, 3, or 4, and Q is OH, $-\text{NHC}(\text{S})\text{N}(\text{R})_2$, $-\text{NHC}(\text{O})\text{N}(\text{R})_2$, $-\text{N}(\text{R})\text{C}(\text{O})\text{R}$, $-\text{N}(\text{R})\text{S}(\text{O})_2\text{R}$, $-\text{N}(\text{R})\text{R}_8$, $-\text{NHC}(=\text{NR}_9)\text{N}(\text{R})_2$, $-\text{NHC}(=\text{CHR}_9)\text{N}(\text{R})_2$, $-\text{OC}(\text{O})\text{N}(\text{R})_2$, $-\text{N}(\text{R})\text{C}(\text{O})\text{OR}$, het-

65

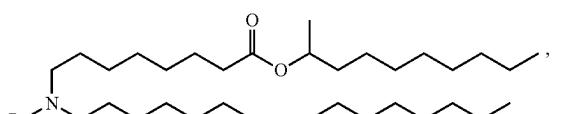
eroaryl or heterocycloalkyl; M and M' are independently selected from $-\text{C}(\text{O})\text{O}-$, $-\text{OC}(\text{O})-$, $-\text{C}(\text{O})\text{N}(\text{R}')-$, $-\text{P}(\text{O})(\text{OR}')\text{O}-$, $-\text{S}-\text{S}-$, an aryl group, and a heteroaryl group; and R₂ and R₃ are independently selected from the group consisting of H, C₁₋₁₄ alkyl, and C₂₋₁₄ alkenyl.

In some embodiments, the compound of Formula (X) is of Formula (XIa), (XIb), (XIc), or (XIe):

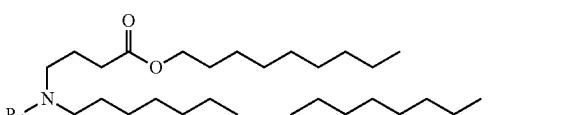
10 (XIa)



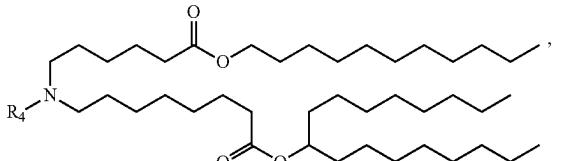
15 (XIb)



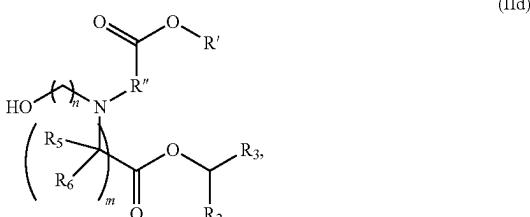
20 (XIc)



25 (XIe)

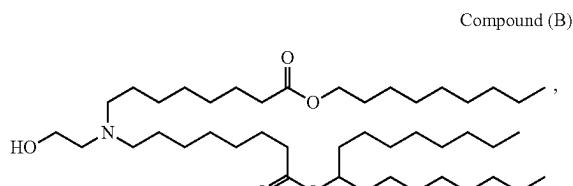
45 or a salt or isomer thereof, wherein R₄ is as described herein.

In certain embodiments, a compound of Formula (X) is of Formula (XId):



50 or a salt or isomer thereof, wherein n is 2, 3, or 4; and m, R', R'', and R₂ through R₆ are as described herein. For example, each of R₂ and R₃ may be independently selected from the group consisting of C₅₋₁₄ alkyl and C₅₋₁₄ alkenyl.

In certain embodiments, the compound of Formula (X) has the following structure:

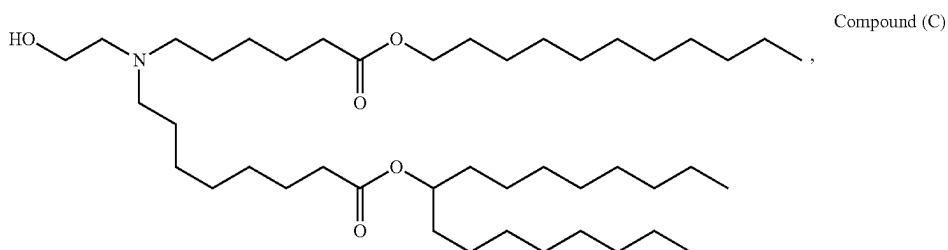
49

or a salt thereof.

50

In certain embodiments, the LNP comprises a high-purity PEG lipid component described herein, and compound (B). In certain embodiments, the LNP comprises a plurality of compounds of Formula (I) described herein, and compound (B).

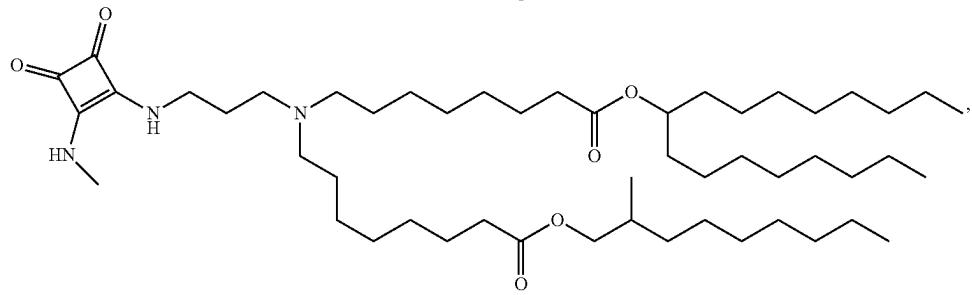
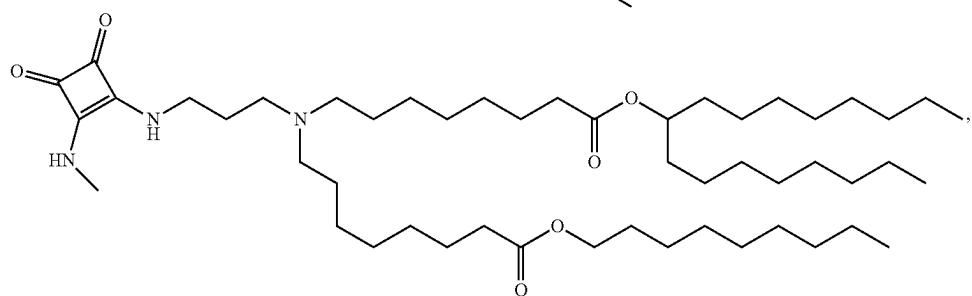
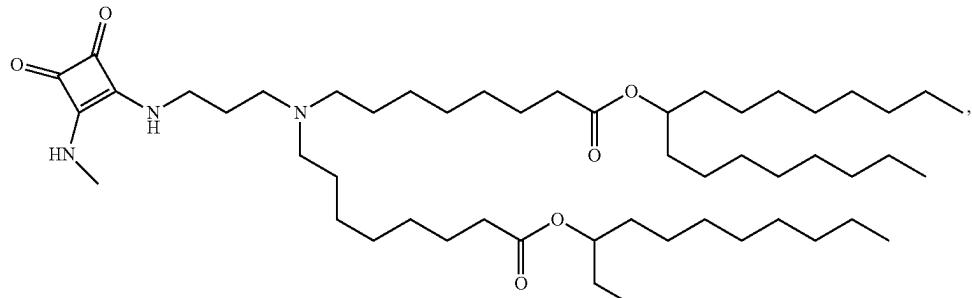
In certain embodiments, the compound of Formula (X) has the structure:



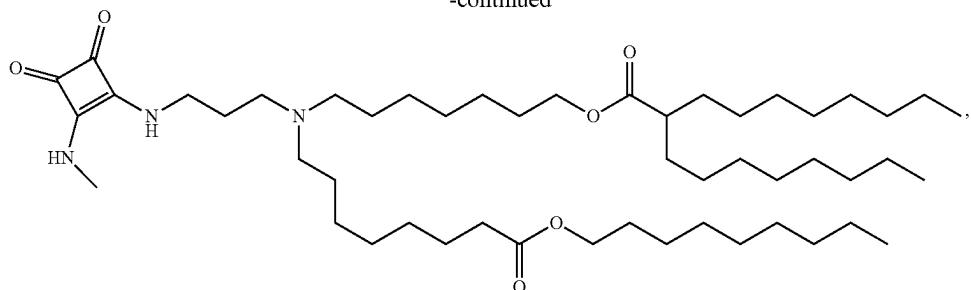
or a salt thereof.

²⁵ In certain embodiments, the LNP comprises a high-purity PEG lipid component described herein, and compound (C). In certain embodiments, the LNP comprises a plurality of compounds of Formula (I) described herein, and compound (C).

³⁰ In certain embodiments, a compound of Formula (X) is selected from the group consisting of:



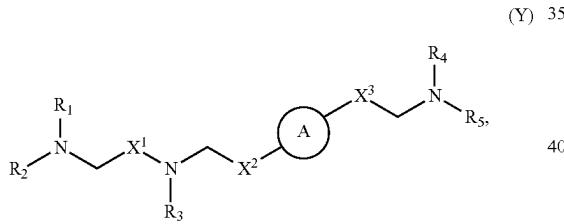
-continued



and pharmaceutically acceptable salts thereof. In certain embodiments, the LNP comprises a high-purity PEG lipid component described herein, and one of the preceding compounds of Formula (X). In certain embodiments, the LNP comprises a plurality of compounds of Formula (I) described herein, one of the preceding compounds of Formula (X).

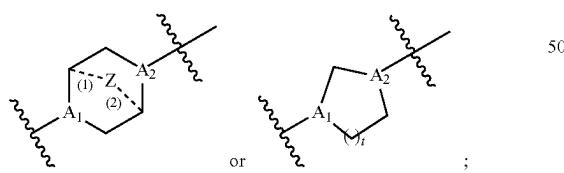
In certain embodiments, the LNP comprises a high-purity PEG lipid component described herein (i.e., with the proscribed/recommended purity of 87% or greater with respect to the PEG lipid component), and a compound of Formula (Y). In certain embodiments, the LNP comprises a plurality of compounds of Formula (I) described herein (i.e., with the proscribed/recommended purity of 87% or greater with respect to the PEG lipid component) and a compound of Formula (Y).

A compound of Formula (Y) has the following formula:



or a salt thereof, wherein:

Ring A is



A_1 and A_2 are each independently selected from CH and N;

Z is CH_2 or absent wherein when Z is CH_2 , the dashed lines (1) and (2) each represent a single bond; and when Z is absent, the dashed lines (1) and (2) are both absent; R_1 , R_2 , R_3 , R_4 , and R_5 are independently selected from the group consisting of C_{5-20} alkyl, C_{5-20} alkenyl, $-R''MR'$, $-R''YR'$, $-YR'$, and $-R''OR'$; each M is independently selected from the group consisting of $-C(O)O-$, $-OC(O)-$, $-OC(O)O-$, $-C(O)N(R')-$, $-N(R')C(O)-$, $-C(O)-$, $-C(S)-$,

$-C(S)S-$, $-SC(S)-$, $-CH(OH)-$, $-P(O)(OR')$ $O-$, $-S(O)_2-$, an aryl group, and a heteroaryl group; X_1 , X_2 , and X_3 are independently selected from the group consisting of a bond, $-CH_2-$, $-(CH_2)_2-$, $-CHR-$, $-CHY-$, $-C(O)-$, $-C(O)O-$, $-OC(O)-$, $-C(O)-CH_2-$, $-CH_2C(O)-$, $-C(O)O-CH_2-$, $-OC(O)-CH_2-$, $-CH_2C(O)O-$, $-CH_2OC(O)-$, $-CH(OH)-$, $-C(S)-$, and $-CH(SH)-$;

each Y is independently a C_{3-6} carbocycle;

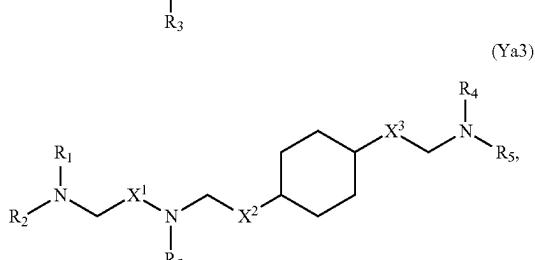
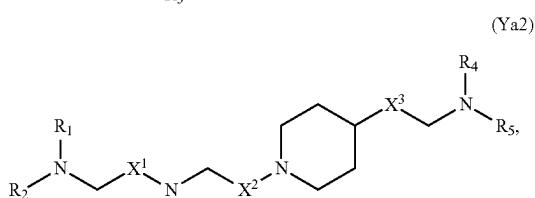
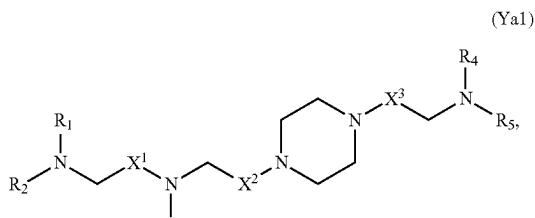
each R'' is independently selected from the group consisting of C_{1-12} alkyl and C_{2-12} alkenyl;

each R is independently selected from the group consisting of C_{1-3} alkyl and a C_{3-6} carbocycle;

each R' is independently selected from the group consisting of C_{1-12} alkyl, C_{2-12} alkenyl, and H; and

each R'' is independently selected from the group consisting of C_{3-12} alkyl and C_{3-12} alkenyl.

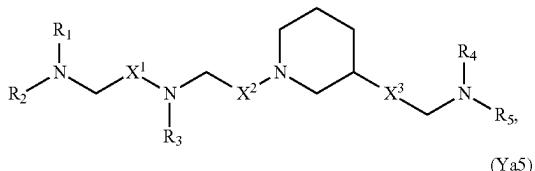
In certain embodiments, the compound of Formula (Y) is of one of the Formulae (Ya1), (Ya2), (Ya3), (Ya4), (Ya5), or (Ya6):



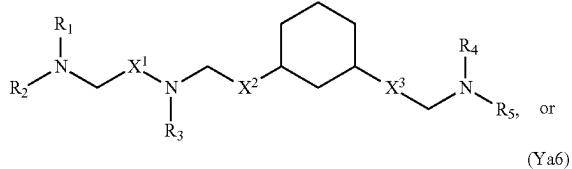
53

-continued

(Ya4)



(Ya5)



5

10

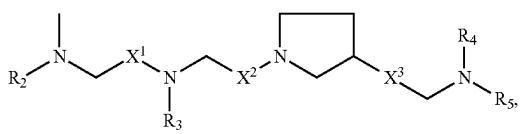
15

15

20

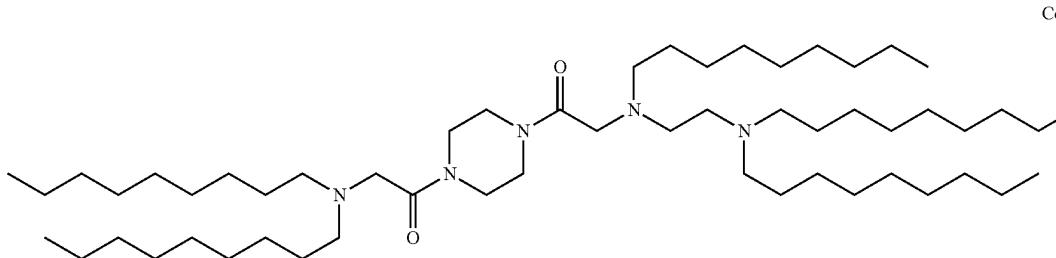
embodiments, the ionizable amino lipid is present in a molar ratio of about 25-35% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 30-40% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 35-45% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 40-50% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 45-55% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, or 45% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 30% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 40% with respect to other lipids.

Examples of ionizable amino lipids (also called “cationic lipids”) are provided herein. Ionizable/cationic lipids generally are lipids that may have a positive or partial positive charge at physiological pH. In certain embodiments, ionizable amino lipids useful in the present invention can be found in International Publication No. WO 2017/049245, published Mar. 23, 2017, or International Publication No.



or a salt or stereoisomer thereof.

In certain embodiments, the compound of Formula (Y) is of the structure:



or a pharmaceutically acceptable salt thereof.

In certain embodiments, the LNP comprises a high-purity PEG lipid component described herein, and compound (A). In certain embodiments, the LNP comprises a plurality of compounds of Formula (I) described herein, and compound (A).

Additional examples of compounds of Formulae (X) and (Y) can be found in, for example, International Publication No. WO 2017/049245, published Mar. 23, 2017, and International Publication No. WO 2017/112865, the entire contents of each of which is incorporated herein by reference.

In certain embodiments, the ionizable amino lipid (e.g., a compound of Formula (X) or (Y), or Compound (A), (B), or (C)) is present in a molar ratio of about 25-65% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 25-50% with respect to other lipids.

In certain embodiments, the ionizable amino lipid (e.g., a compound of Formula (X) or (Y), or Compound (A), (B), or (C)) is present in a molar ratio of about 40% or less with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 35% or less with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 30% or less with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 25-40% with respect to other lipids. In certain

40 WO 2017/112865, the entire contents of each of which is incorporated herein by reference.

In certain embodiments, cationic and/or ionizable lipids may be selected from the non-limiting group consisting of 3-(didodecylamino)-N1,N1,4-tridodecyl-1-piperazineethanamine (KL10), N1-[2-(didodecylamino)ethyl]N1,N4,N4-tridodecyl-1,4-piperazinediethanamine (KL22), 14,25-ditridodecyl-15,18,21,24-tetraaza-octatriacontane (KL25), 1,2-dilinoleyoxy-N,N-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA), 2,2-dilinoleyl-4-(2 dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 2-{8 [(3β)-cholest-5-en-3-yloxy]octyl}oxy N,N dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA), (2R)-2-{8-[(3p)-cholest-5-en-3-yloxy]octyl}oxy-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2R)), and (2S) 2-{8-[(3p)-cholest-5-en-3-yloxy]octyl}oxy-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Octyl-CLinDMA (2S)). In addition to these, a cationic lipid may also be a lipid including a cyclic amine group. Each possibility represents a separate embodiment of the present invention. Each possibility represents a separate embodiment of the present invention.

In one embodiment, the cationic lipid may be selected from, but not limited to, a cationic lipid described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865, WO2008103276, WO2013086373 and WO2013086354, U.S. Pat. Nos. 7,893,302, 7,404,969, 8,283,333, and 8,466,122 and US Patent Publication No. US20100036115, US20120202871, US20130064894, US20130129785, US20130150625, US20130178541 and S20130225836; the contents of each of which are herein incorporated by reference in their entirety.

In another embodiment, the cationic lipid may be selected from, but not limited to, formula A described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638 and WO2013116126 or US Patent Publication No. US20130178541 and US20130225836; the contents of each of which is herein incorporated by reference in their entirety. In yet another embodiment, the cationic lipid may be selected from, but not limited to, formula CLI-CLXXIX of International Publication No. WO2008103276, formula CLI-CLXXIX of U.S. Pat. No. 7,893,302, formula CLI-CLXXXII of U.S. Pat. No. 7,404,969 and formula I-VI of US Patent Publication No. US20100036115, formula I of US Patent Publication No US20130123338; each of which is herein incorporated by reference in their entirety.

As a non-limiting example, the cationic lipid may be selected from (20Z,23Z)—N,N-dimethylnonacos-20,23-dien-10-amine, (17Z,20Z)—N,N-dimethylhexacos-17,20-dien-9-amine, (1Z,19Z)—N5N-dimethylpentacosa-16, 19-dien-8-amine, (13Z,16Z)—N,N-dimethyldocosa-13,16-dien-5-amine, (12Z,15Z)—N,N dimethylhenicos-12,15-dien-4-amine, (14Z,17Z)—N,N-dimethyltricos-14,17-dien-6-amine, (15Z,18Z)—N,N-dimethyltetracos-15,18-dien-7-amine, (18Z,21Z)—N,N-dimethylheptacosa-18,21-dien-10-amine, (15Z,18Z)—N,N-dimethyltetraicos-15,18-dien-5-amine, (14Z,17Z)—N,N-dimethyltricos-14,17-dien-4-amine, (19Z,22Z)—N,N-dimeihyloctacos-19,22-dien-9-amine, (18Z,21 Z)—N,N-dimethylheptacosa-18,21-dien-8-amine, (17Z,20Z)—N,N-dimethylhexacos-17,20-dien-7-amine, (16Z,19Z)—N,N-dimethylpentacos-16,19-dien-6-amine, (22Z,25Z)—N,N-dimethylhentriaconta-22, 25-dien-10-amine, (21 Z,24Z)—N,N-dimethyltriaconta-21, 24-dien-9-amine, (18Z)—N,N-dimethylheptacos-18-en-10-amine, (17Z)—N,N-dimethylhexacos-17-en-9-amine, (19Z, 22Z)—N,N-dimethyloctacos-19,22-dien-7-amine, N,N-dimethylheptacosan-10-amine, (20Z,23Z)—N-ethyl-N-methylnonacos-20,23-dien-10-amine, 1-[(11Z,14Z)-1-nonylicosa-11,14-dien-1-yl] pyrrolidine, (20Z)—N,N-dimethylheptacos-20-en-10-amine, (15Z)—N,N-dimethyl eptacos-15-en-10-amine, (14Z)—N,N-dimethylnonacos-14-en-10-amine, (17Z)—N,N-dimethylnonacos-17-en-10-amine, (24Z)—N,N-dimethyltritriacont-24-en-10-amine, (20Z)—N,N-dimethylnonacos-20-en-10-amine, (22Z)—N, N-dimethylhentriacont-22-en-10-amine, (16Z)—N,N-dimethylpentacos-16-en-8-amine, (12Z,15Z)—N,N-dimethyl-2-nonylhenicos-12,15-dien-1-amine, (13Z,16Z)—N,N-dimethyl-3-nonyldocosa-13,16-dien-1-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl] eptadecan-8-amine, 1-[(1S,2R)-2-hexylcyclopropyl]-N,N-dimethylnonadecan-10-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]nonadecan-10-amine, N,N-dimethyl-21-[(1S,2R)-2-octylcyclopropyl]henicosan-10-amine, N,N-dimethyl-1-[(1S,2S)-2-[(1R,2R)-2-pentylcyclopropyl]

methyl}cyclopropyl]nonadecan-10-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]hexadecan-8-amine, N,N-dimethyl-1-[(1R,2S)-2 undecylcyclopropyl]tetradecan-5-amine, N,N-dimethyl-3-[(7-[(1S,2R)-2-octylcyclopropyl]heptyl) dodecan-1-amine, 1-[(1R,2S)-2-heptylcyclopropyl]-N,N-dimethyloctadecan-9-amine, 1-[(1S,2R)-2-decylcyclopropyl]-N,N-dimethylpentadecan-6-amine, N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]pentadecan-8-amine, R—N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, S—N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}pyrrolidine, (2S)—N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-[(5Z)-oct-5-en-1-yloxy]propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}azetidine, (2S)-1-(hexyloxy)-N,N-dimethyl-3-[(9Z, 12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2S)-1-(heptyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(9Z)-octadec-9-en-1-yloxy]-3-(octyloxy)propan-2-amine; (2S)—N,N-dimethyl-1-[(6Z,9Z,12Z)-octadeca-6,9,12-trien-1-yloxy]-3-(octyloxy)propan-2-amine, (2S)-1-[(11Z,14Z)-icos-11,14-dien-1-yloxy]-N,N-dimethyl-3-(pentyloxy)propan-2-amine, (2S)-1-(hexyloxy)-3-[(11Z,14Z)-icos-11,14-dien-1-yloxy]-N,N-dimethylpropan-2-amine, 1-[(11Z,14Z)-icos-11,14-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2S)-1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, (2S)-1-[(13Z)-docos-13-en-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, 1-[(13Z)-docos-13-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(9Z)-hexadec-9-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2R)—N,N-dimethyl-H(1-methyloctyl)oxy]-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2R)-1-[(3,7-dimethyloctyl)oxy]-N,N-dimethyl-3-[(9Z, 12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(8-[(1S,2S)-2-[(1R,2R)-2-pentylcyclopropyl]methyl]cyclopropyl]oxy]propan-2-amine, N,N-dimethyl-1-[(8-(2-octylcyclopropyl)octyl)oxy]propan-2-amine and (11E,20Z,23Z)—N,N-dimethylnonacos-11,20,2-trien-10-amine or a pharmaceutically acceptable salt or stereoisomer thereof. Each possibility represents a separate embodiment of the present invention.

In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012170889, herein incorporated by reference in its entirety. In another embodiment, the lipid may be a cationic lipid such as, but not limited to, Formula (I) of U.S. Patent Application No. US20130064894, the contents of which are herein incorporated by reference in its entirety. In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2013086354; the contents of each of which are herein incorporated by reference in their entirety. In another embodiment, the cationic lipid may be a trialkyl cationic lipid. Non-limiting examples of trialkyl cationic lipids and methods of making and using the trialkyl cationic lipids are described in International Patent Publication No. WO2013126803, the contents of which are herein incorporated by reference in its entirety.

In some embodiments, LNPs comprise the lipid KL52 (an amino-lipid disclosed in U.S. Application Publication No.

2012/0295832 incorporated herein by reference in its entirety). Activity and/or safety (as measured by examining one or more of ALT/AST, white blood cell count and cytokine induction) of LNP administration may be improved by incorporation of such lipids. LNPs comprising KL52 may be administered intravenously and/or in one or more doses. In some embodiments, administration of LNPs comprising KL52 results in equal or improved mRNA and/or protein expression as compared to LNPs comprising MC3.

As a non-limiting example, the LNP may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine and the cationic peptides described in International Pub. No. WO2012013326 or US Patent Pub. No. US20130142818; each of which is herein incorporated by reference in its entirety.

Other LNP Components

In certain embodiments, the LNP comprises a helper lipid. In certain embodiments, the helper lipid is present in a molar ratio of about 10-40% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 10-20% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 15-25% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 20-30% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 25-35% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 30-40% with respect to other lipids. In certain embodiments, the ionizable amino lipid is present in a molar ratio of about 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, or 35% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 10% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 20% with respect to other lipids. In certain embodiments, the helper lipid is present in a molar ratio of about 30% with respect to other lipids.

Examples of helper lipids are provided herein. In certain embodiments, the helper lipid is a phospholipid. In certain embodiments, the helper lipid is DSPC or an analog thereof. In certain embodiments, the helper lipid is DOPE. In certain embodiments, the helper lipid is oleic acid or an analog thereof. In certain embodiments, the helper lipid is oleic acid.

Helper lipids useful in the present invention include neutral and/or non-cationic lipids (e.g., lipids that are neutral or non-cationic lipid at physiological pH). Helper lipids may also be referred to as “non-cationic” lipids. In certain embodiments, the helper lipid is a phospholipid.

For other embodiments, including examples of helper lipids useful in the present invention, see International Publication No. WO 2017/099823, published Jun. 15, 2017, the entire contents of which is incorporated herein by reference.

Phospholipids, as defined herein, are any lipids that comprise a phosphate group. Phospholipids are a subset of non-cationic lipids. The lipid component of a nanoparticle composition may include one or more phospholipids, such as one or more (poly)unsaturated lipids. Phospholipids may assemble into one or more lipid bilayers. In general, phospholipids may include a phospholipid moiety and one or more fatty acid moieties. A phospholipid moiety may be selected from the non-limiting group consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, 2-lyso-

phosphatidyl choline, and a sphingomyelin. A fatty acid moiety may be selected from the non-limiting group consisting of lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, erucic acid, phytanoic acid, arachidic acid, arachidonic acid, eicosapentaenoic acid, behenic acid, docosapentaenoic acid, and docosahexaenoic acid. Non-natural species including natural species with modifications and substitutions including branching, oxidation, cyclization, and alkynes are also contemplated. For example, a phospholipid may be functionalized with or cross-linked to one or more alkynes (e.g., an alkenyl group in which one or more double bonds is replaced with a triple bond). Under appropriate reaction conditions, an alkyne group may undergo a copper-catalyzed cycloaddition upon exposure to an azide. Such reactions may be useful in functionalizing a lipid bilayer of a nanoparticle composition to facilitate membrane permeation or cellular recognition or in conjugating a nanoparticle composition to a useful component such as a targeting or imaging moiety (e.g., a dye). Each possibility represents a separate embodiment of the present invention.

Helper lipids, in particular phospholipids, useful in the compositions and methods may be selected from the non-limiting group consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-(1-glycerol) sodium salt (DOPG), and sphingomyelin. Each possibility represents a separate embodiment of the present invention. In some embodiments, a nanoparticle composition includes DSPC. In certain embodiments, a nanoparticle composition includes DOPE. In some embodiments, a nanoparticle composition includes both DSPC and DOPE.

In certain embodiments, the helper lipid is a fatty acid. The fatty acid may be saturated or unsaturated. Examples of unsaturated fatty acids include, but are not limited to, myristoleic acid, palmitoleic acid sapienic acid, oleic acid, elaidic acid, vaccenic acid, linoleic acid, linoelaidic acid, alpha-linoelaidic acid arachidonic acid, eicosapentaenoic acid, erucic acid, docosahexanoic acid, or any cis/trans double-bond isomers thereof. In certain embodiments, the lipid is oleic acid. In certain embodiments, the lipid is an isomer of oleic acid (e.g., the double bond is in a different location along the aliphatic chain relative to oleic acid). In certain embodiments, the lipid is an analog of oleic acid (e.g., the aliphatic chain is 1-10 carbons longer or 1-10 carbons shorter than the aliphatic chain of oleic acid). Examples of saturated fatty acids include, but are not limited

to, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid, and cerotic acid. In certain embodiments, an oleic acid analog is a compound wherein the carboxylic acid moiety of oleic acid replaced by a different group.

In certain embodiments, the helper lipid is a glycine derivative of a fatty acid (e.g., N-palmitoylglycine or N-oleylglycine). In certain embodiments, the helper lipid is a glycerolipid (e.g., monoglyceride, diglyceride, triglyceride). In certain embodiments, the helper lipid is a monoglyceride. In certain embodiments, the helper lipid is a diglyceride. In certain embodiments, the helper lipid is a triglyceride. In certain embodiments, the helper lipid comprises a sugar moiety (e.g., saccharide, disaccharide, polysaccharide).

In certain embodiments, the LNP further comprises a structural lipid. In certain embodiments, the structural lipid is present in a molar ratio of about 30-50% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 30-40% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 35-45% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 40-50% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 30-35% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 35-40% with respect to other lipids. In certain embodiments, the structural lipid is

ments, the structural lipid is an analog of cholesterol. In certain embodiments, the structural lipid is alpha-tocopherol.

For other embodiments, including examples of helper lipids useful in the present invention, see International Publication No. WO 2017/099823, published Jun. 15, 2017, the entire contents of which is incorporated herein by reference.

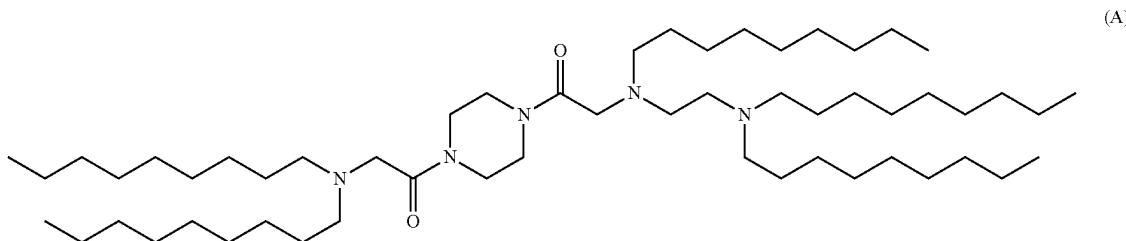
In certain embodiments, the LNP comprises a plurality of 10 PEG lipids provided herein (e.g., a plurality of compounds of Formula (I) provided herein), an ionizable amino lipid, a helper lipid, and a structural lipid.

In certain embodiments, the LNP comprises 15-65% ionizable amino lipid, about 0.15-15% PEG lipids provided herein (e.g., a plurality of compounds of Formula (I) provided herein), about 30-50% structural lipid, and about 10-40% helper lipid.

Many other lipids and LNP formulations applicable to the present disclosure can be found in, e.g., International Publication No. WO 2017/099823, published Jun. 15, 2017, the entire contents of which is incorporated herein by reference. Certain Embodiments of Lipid Nanoparticles (LNPs)

Provided herein are lipid nanoparticles (LNPs) comprising an ionizable amino lipid, a PEG lipid, a helper lipid (e.g., phospholipid), and a structural lipid. In certain embodiments, the LNP comprises an agent to be delivered.

In certain embodiments, an LNP provided herein comprises the ionizable amino lipid Compound (A):



present in a molar ratio of about 40-45% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 45-50% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 35%, 35.5%, 36%, 36.5%, 37%, 37.5%, 38%, 38.5%, 39%, 39.5%, 40%, 40.5%, 41%, 41.5%, or 42% with respect to other lipids. In certain embodiments, the structural lipid is present in a molar ratio of about 38.5% with respect to other lipids.

Examples of structural lipids are provided herein. In certain embodiments, the structural lipid is a sterol. In certain embodiments, the structural lipid is cholesterol.

Non-limiting examples of structural lipids useful in the invention are provided herein. Incorporation of structural lipids in the lipid nanoparticle may help mitigate aggregation of other lipids in the particle. Structural lipids can be selected from the group consisting of, but are not limited to, cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, tomatine, ursolic acid, alpha-tocopherol, hopanoids, phytosterols, steroids, and mixtures thereof. In some embodiments, the structural lipid is a sterol. As defined herein, "sterols" are a subgroup of steroids consisting of steroid alcohols. In certain embodiments, the structural lipid is a steroid. In certain embodiments, the structural lipid is cholesterol. In certain embodi-

a PEG lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (helper lipid), and cholesterol (structural lipid).

In certain embodiments, the LNP comprises Compound (A), high-purity PEG lipids as described herein (i.e., having a purity of 87% or greater as described herein), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol. In certain embodiments, the LNP comprises Compound (A), a plurality of compounds of Formula (I) as described herein (i.e., having a purity of 87% or greater as described herein), DOPE, and cholesterol.

In certain embodiments, Compound (A) is present in a molar ratio of 40% or less with respect to the other lipids. In certain embodiments, Compound (A) is present in a molar ratio of 20-40%, inclusive, with respect to the other lipids. In certain embodiments, Compound (A) is present in a molar ratio of 25-35%, inclusive, with respect to the other lipids. In certain embodiments, Compound (A) is present in a molar ratio of approximately 30% with respect to other lipids. In certain embodiments, Compound (A) is present in a molar ratio of approximately 40% with respect to other lipids.

In certain embodiments, the PEG lipids are present in a molar ratio of 0.15-15%, inclusive, with respect to the other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of 1-2%, inclusive, with respect to the other

61

lipids. In certain embodiments, the PEG lipids are present in a molar ratio of approximately 1.5% with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 0.15-15%, inclusive, with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 1-2%, inclusive, with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of approximately 1.5% with respect to the other lipids.

In certain embodiments, DOPE is present in a molar ratio of 20-40%, inclusive, with respect to the other lipids. In certain embodiments, DOPE is present in a molar ratio of 25-35%, inclusive, with respect to the other lipids. In certain embodiments, DOPE is present in a molar ratio of approximately 30% with respect to the other lipids.

In certain embodiments, cholesterol is present in a molar ratio of 30-50%, inclusive, with respect to the other lipids. In certain embodiments, cholesterol is present in a molar ratio of 35-40%, inclusive, with respect to the other lipids. In certain embodiments, cholesterol is present in a molar ratio of approximately 38.5% with respect to the other lipids.

In certain embodiments, an LNP provided herein comprises a molar ratio of 40% or less of Compound (A), 0.15-15% of a plurality of compounds of Formula (I) as described herein, 20-40% DOPE, and 30-50% cholesterol. In certain embodiments, an LNP provided herein comprises a molar ratio of 20-40% of Compound (A), 0.15-15% of a plurality of compounds of Formula (I) as described herein, 20-40% DOPE, and 30-50% cholesterol. In certain embodiments, the LNP comprises a molar ratio of 25-35% Compound (A), 1-2% of a plurality of compounds of Formula (I), 25-35% DOPE, and 35-42% cholesterol.

In certain embodiments, the LNP comprises Compound (A):compounds of Formula (I):DOPE:cholesterol in a molar ratio of approximately 30:30:38.5:1.5. In certain embodiments, the LNP comprises Compound (A):compounds of Formula (I):DOPE:cholesterol in a molar ratio of approximately 40:20:38.5:1.5.

Also provided herein are LNPs comprising ionizable amino lipid Compound (B):

62

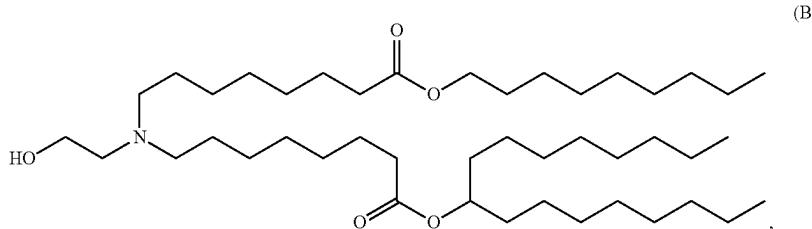
lipids. In certain embodiments, Compound (B) is present in a molar ratio of 40% or less with respect to the other lipids. In certain embodiments, Compound (B) is present in a molar ratio of 20-40%, inclusive, with respect to the other lipids. In certain embodiments, Compound (B) is present in a molar ratio of 25-35%, inclusive, with respect to the other lipids. In certain embodiments, Compound (B) is present in a molar ratio of approximately 30%. In certain embodiments, Compound (B) is present in a molar ratio of approximately 40%.

In certain embodiments, the PEG lipids are present in a molar ratio of 0.15-15%, inclusive, with respect to the other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of 1-2%, inclusive, with respect to the other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of approximately 1.5% with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 0.15-15%, inclusive, with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 1-2%, inclusive, with respect to the other lipids. In certain embodiments, the compound of Formula (I) is present in a molar ratio of approximately 1.5% with respect to the other lipids.

In certain embodiments, DOPE is present in a molar ratio of 20-40%, inclusive, with respect to the other lipids. In certain embodiments, DOPE is present in a molar ratio of 25-35%, inclusive, with respect to the other lipids. In certain embodiments, DOPE is present in a molar ratio of approximately 30% with respect to the other lipids.

In certain embodiments, cholesterol is present in a molar ratio of 30-50%, inclusive, with respect to the other lipids. In certain embodiments, cholesterol is present in a molar ratio of 35-40%, inclusive, with respect to the other lipids. In certain embodiments, cholesterol is present in a molar ratio of approximately 38.5% with respect to the other lipids.

In certain embodiments, the LNP comprises a molar ratio of 40% or less of Compound (B), 0.15-15% of a plurality of compounds of Formula (I), 20-40% DOPE, and 30-50% cholesterol. In certain embodiments, the LNP comprises a molar ratio of 20-40% of Compound (B), 0.15-15% of a plurality of compounds of Formula (I), 20-40% DOPE, and



a PEG lipid, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (helper lipid), and cholesterol (structural lipid).

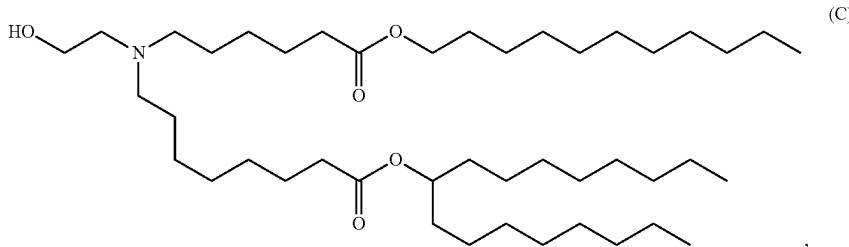
In certain embodiments, the LNP comprises Compound (B), high-purity PEG lipids as described herein (i.e., having a purity of 87% or greater as described herein), DSPC, and cholesterol. In certain embodiments, the LNP comprises Compound (B), a plurality of compounds of Formula (I) as described herein (i.e., having a purity of 87% or greater as described herein), DSPC, and cholesterol.

In certain embodiments, Compound (B) is present in a molar ratio of 20-50%, inclusive, with respect to the other lipids. In certain embodiments, Compound (B) is present in a molar ratio of 30-45%, inclusive, with respect to the other

lipids. In certain embodiments, the LNP comprises a molar ratio of 25-35% Compound (B), 1-2% of a plurality of compounds of Formula (I), 25-35% DOPE, and 35-42% cholesterol.

In certain embodiments, the LNP comprises Compound (B):compounds of Formula (I):DOPE:cholesterol in a molar ratio of approximately 30:30:38.5:1.5. In certain embodiments, the LNP comprises Compound (B):Compounds of Formula (I):DOPE:cholesterol in a molar ratio of approximately 40:20:38.5:1.5.

Also provided herein are LNPs comprising ionizable amino lipid Compound (C):



a PEG lipid, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (helper lipid), and cholesterol (structural lipid).

In certain embodiments, the LNP comprises Compound (C), high-purity PEG lipids described herein (i.e., having a purity of 87% or greater as described herein), DSPC, and cholesterol. In certain embodiments, the LNP comprises Compound (C), a plurality of compounds of Formula (I) described herein (i.e., having a purity of 87% or greater as described herein), DSPC, and cholesterol.

In certain embodiments, Compound (C) is present in a molar ratio of 20-50%, inclusive, with respect to the other lipids. In certain embodiments, Compound (C) is present in a molar ratio of 30-45%, inclusive, with respect to the other lipids. In certain embodiments, Compound (C) is present in a molar ratio of 40% or less with respect to the other lipids. In certain embodiments, Compound (C) is present in a molar ratio of 20-40%, inclusive, with respect to the other lipids. In certain embodiments, Compound (C) is present in a molar ratio of 25-35%, inclusive, with respect to the other lipids. In certain embodiments, Compound (C) is present in a molar ratio of approximately 30% with respect to the other lipids. In certain embodiments, Compound (C) is present in a molar ratio of approximately 40% with respect to the other lipids.

In certain embodiments, the PEG lipids are present in a molar ratio of 0.15-15%, inclusive, with respect to the other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of 1-2%, inclusive, with respect to the other lipids. In certain embodiments, the PEG lipids are present in a molar ratio of approximately 1.5% with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 0.15-15%, inclusive, with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of 1-2%, inclusive, with respect to the other lipids. In certain embodiments, the plurality of compounds of Formula (I) is present in a molar ratio of approximately 1.5% with respect to the other lipids.

In certain embodiments, DOPE is present in a molar ratio of 20-40%, inclusive, with respect to the other lipids. In certain embodiments, DOPE is present in a molar ratio of 25-35%, inclusive, with respect to the other lipids. In certain embodiments, DOPE is present in a molar ratio of approximately 30% with respect to the other lipids.

In certain embodiments, cholesterol is present in a molar ratio of 30-50%, inclusive, with respect to the other lipids. In certain embodiments, cholesterol is present in a molar ratio of 35-40%, inclusive, with respect to the other lipids. In certain embodiments, cholesterol is present in a molar ratio of approximately 38.5% with respect to the other lipids.

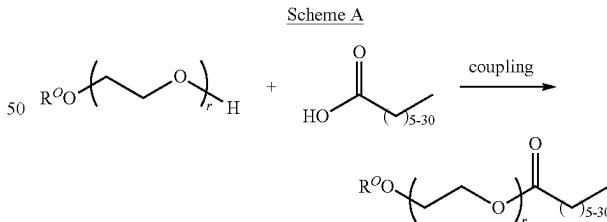
In certain embodiments, the LNP comprises a molar ratio of 40% or less of Compound (C), 0.15-15% of a plurality of compounds of Formula (I), 20-40% DOPE, and 30-50% cholesterol. In certain embodiments, the LNP comprises a molar ratio of 20-40% of Compound (C), 0.15-15% of a plurality of compounds of Formula (I), 20-40% DOPE, and 30-50% cholesterol. In certain embodiments, the LNP comprises a molar ratio of 25-35% Compound (C), 1-2% of a plurality of compounds of Formula (I), 25-35% DOPE, and 35-42% cholesterol.

In certain embodiments, the LNP comprises Compound (C):compounds of Formula (I):DOPE:cholesterol in a molar ratio of approximately 30:30:38.5:1.5. In certain embodiments, the LNP comprises Compound (C):compounds of Formula (I):DOPE:cholesterol in a molar ratio of approximately 40:20:38.5:1.5.

Methods for Preparing PEG Lipids

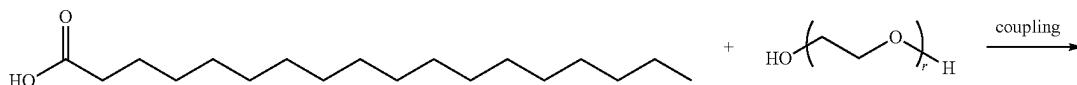
Provided herein are methods for preparing PEG lipids, including compounds of Formula (I). As shown in Scheme A and Scheme B, the methods comprise reacting a PEG component with a carboxylic acid component to yield a PEG lipid.

45

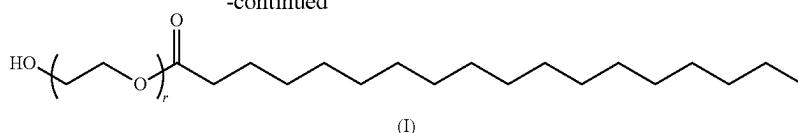


55

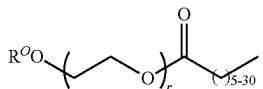
Scheme B



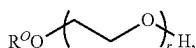
-continued



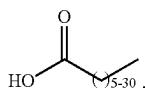
Provided herein are methods of preparing a compound of the formula:



or a salt thereof, the method comprising reacting a compound of the formula:

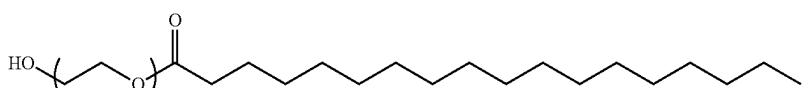


or a salt thereof, with a compound of the formula:



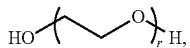
or a salt thereof; wherein r is an integer from 35-55, inclusive; and R' is hydrogen, optionally substituted alkyl, or an oxygen protecting group.

In certain embodiments, the method is a method for preparing a compound of Formula (I):

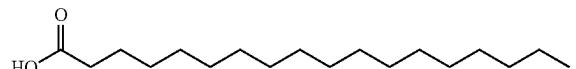


45

or a salt thereof, the method comprising reacting a compound of the formula:



or a salt thereof, with a compound of the formula:



or a salt thereof; wherein r is an integer from 35-55, inclusive.

In certain embodiments, greater than 1 equivalent of the PEG component is used (with respect to the carboxylic acid component). In certain embodiments, 1-20 equivalents of the PEG component is used. In certain embodiments, 1-10 equivalents of the PEG component is used. In certain

10 embodiments, approximately 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 equivalents of the PEG component is used. In certain embodiments, approximately 2 equivalents of the PEG component is used. In certain embodiments, approximately 10 equivalents of the carboxylic acid component is used.

15 In certain embodiments, the reaction is carried out in the presence of a carboxyl activating reagent. Carboxyl activating reagents are reagents capable of activating carboxyl groups for addition by nucleophiles (e.g., alcohols, amines). Carboxyl activating reagents are known in the art. In certain embodiments, the carboxyl activating reagent is a carbodiimide. In certain embodiments, the reagent is N,N'-dicyclohexylcarbodiimide (DCC). In certain embodiments, the reagent is N,N'-diisopropylcarbodiimide (DIC). In certain embodiments, the reagent is 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). In certain embodiments, the reagent is the hydrochloride salt of the carbodiimide. In certain embodiments, the reagent is the hydrochloride salt of EDC (EDC-HCl). In certain embodiments, greater than 1 equivalent (with respect to the carboxylic acid component) of the carboxyl activating agent (e.g., EDC or salt thereof) is used. In certain embodiments, 1-2 equivalents is used, inclusive. In certain embodiments, approximately 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0 equivalents is used. In certain embodiments, approximately 1.5 equivalents is used.

20 35 In certain embodiments, the reaction is carried out in the presence of a base. In certain embodiments, the base is an amine base. In certain embodiments, the base is a tri-

(I)

50 kylamine base. In certain embodiments, the base is a pyridine base. In certain embodiments, the base is 4-Dimethylaminopyridine (DMAP). In certain embodiments, less than 1 equivalent (with respect to the carboxylic acid component) of the base (e.g., DMAP) is used. In certain embodiments, 0.01-1.0 equivalents is used, inclusive. In certain embodiments, approximately 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 equivalents is used. In certain embodiments, approximately 0.2 equivalents is used.

55 In certain embodiments, the reaction is carried out in the presence of a carboxyl activating reagent and a base. In certain embodiments, the reaction is carried out in the presence of EDC and a base. In certain embodiments, the reaction is carried out in the presence of EDC and DMAP. In certain embodiments, the reaction is carried out in the presence of EDC-HCl and DMAP.

60 In certain embodiments, the reaction is carried out in solvent. Non-limiting examples of solvents are provided below. In certain embodiments, the solvent is dichloromethane (DCM). In certain embodiments, the reaction is carried out at around room temperature. In certain embodiments, the

reaction is carried out at a temperature below room temperature. In certain embodiments, the reaction is carried out at a temperature from approximately 0° C. to approximately room temperature. In certain embodiments, the reaction is carried out at a temperature from approximately 0° C. to approximately 20° C.

In certain embodiments, the reaction is carried out in the presence of EDC·HCl and DMAP, in DCM. In certain embodiments, the reaction is carried out in the presence of EDC·HCl and DMAP, in DCM, at a temperature below room temperature. In certain embodiments, the reaction is carried out in the presence of EDC·HCl and DMAP, in DCM, at a temperature from approximately 0° C. to approximately 20° C. In certain embodiments, the reaction is carried out under the following conditions: 1 equivalent of carboxylic acid and 2.0 equivalents PEG component, in the presence of 1.5 equivalents EDC·HCl and 0.2 equivalents DMAP, in DCM, at a temperature from approximately 0° C. to approximately 20° C.

In certain embodiments, the PEG lipid is purified by extraction (i.e., workup). In certain embodiments, the PEG lipid is purified by a tri-layer extraction. In certain embodiments, the PEG lipid is purified by tri-layer extraction followed by column chromatography. In certain embodiments, the tri-layer extraction involves partitioning a PEG lipid mixture between acetonitrile, water (or an aqueous solution), methyl tert-butyl ether (MTBE), and heptane. In certain embodiments, the step of purifying by column chromatography is reverse phase ultra-performance liquid chromatography (UPLC).

General Reaction Parameters: The following embodiments apply to all synthetic methods (i.e., methods for preparing PEG lipids) described above and herein.

The reactions provided and described herein may involve one or more reagents. In certain embodiments, a reagent may be present in a catalytic amount. In certain embodiments, a catalytic amount is from 0-1 mol %, 0-5 mol %, 0-10 mol %, 1-5 mol %, 1-10 mol %, 5-10 mol %, 10-20 mol %, 20-30 mol %, 30-40 mol %, 40-50 mol %, 50-60 mol %, 60-70 mol %, 70-80 mol %, 80-90 mol %, or 90-99 mol %. In certain embodiments, a reagent may be present in a stoichiometric amount (i.e., about 1 equivalent). In certain embodiments, a reagent may be present in excess amount (i.e., greater than 1 equivalent). In certain embodiments, the excess amount is about 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 15, or 20 equivalents. In certain embodiments, the excess amount is from about 1.1-2, 2-3, 3-4, 4-5, 1.1-5, 5-10, 10-15, 15-20, or 10-20 equivalents. In certain embodiments, the excess amount is greater than 20 equivalents.

A reaction described herein may be carried out at any temperature. In certain embodiments, a reaction is carried out at or around room temperature (rt) (21° C. or 70° F.). In certain embodiments, a reaction is carried out at below room temperature (e.g., from -100° C. to 21° C.). In certain embodiments, a reaction is carried out at or around -78° C. In certain embodiments, a reaction is carried out at or around -10° C. In certain embodiments, a reaction is carried out at around 0° C. In certain embodiments, a reaction is carried out at from approximately -78° C. to approximately room temperature. In certain embodiments, a reaction is carried out at from approximately -10° C. to approximately room temperature. In certain embodiments, a reaction is carried out at approximately 0° C. to approximately room temperature. In certain embodiments, a reaction is carried out at above room temperature. In certain embodiment, a reaction

is carried out at 30, 40, 50, 60, 70, 80, 110, 120, 130, 140, or 150° C. In certain embodiments, a reaction is carried out at above 150° C.

A reaction described herein may be carried out in a solvent, or a mixture of solvents (i.e., cosolvents). Solvents can be polar or non-polar, protic or aprotic. Any solvent may be used in the reactions described herein, and the reactions are not limited to particular solvents or combinations of solvents. Common organic solvents useful in the methods described herein include, but are not limited to, acetone, acetonitrile, benzene, benzonitrile, 1-butanol, 2-butanone, butyl acetate, tert-butyl methyl ether, carbon disulfide carbon tetrachloride, chlorobenzene, 1-chlorobutane, chloroform, cyclohexane, cyclopentane, 1,2-dichlorobenzene, 1,2-dichloroethane, dichloromethane (DCM), N,N-dimethylacetamide N,N-dimethylformamide (DMF), 1,3-dimethyl-3,4,5,6-tetrahydro-2-pyrimidinone (DMPU), 1,4-dioxane, 1,3-dioxane, diethylether, 2-ethoxyethyl ether, ethyl acetate, ethyl alcohol, ethylene glycol, dimethyl ether, heptane, n-hexane, hexanes, hexamethylphosphoramide (HMPA), 2-methoxyethanol, 2-methoxyethyl acetate, methyl alcohol, 2-methylbutane, 4-methyl-2-pentanone, 2-methyl-1-propanol, 2-methyl-2-propanol, 1-methyl-2-pyrrolidinone, dimethylsulfoxide (DMSO), nitromethane, 1-octanol, pentane, 3-pentanone, 1-propanol, 2-propanol, pyridine, tetrachloroethylene, tetrahydofuran (THF), 2-methyltetrahydrofuran, toluene, trichlorobenzene, 1,1,2-trichlorotrifluoroethane, 2,2,4-trimethylpentane, trimethylamine, triethylamine, N,N-diisopropylethylamine, diisopropylamine, water, o-xylene, p-xylene.

A reaction described herein may be carried out over any amount of time. In certain embodiments, a reaction is allowed to run for seconds, minutes, hours, or days.

Methods described herein can be used to prepare compounds in any chemical yield. In certain embodiments, a compound is produced in from 1-10%, 10-20% 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, or 90-100% yield. In certain embodiments, the yield is the percent yield after one synthetic step. In certain embodiments, the yield is the percent yield after more than one synthetic step (e.g., 2, 3, 4, or 5 synthetic steps).

Methods described herein may further comprise one or more purification steps. For example, in certain embodiments, a compound produced by a method described herein may be purified by chromatography, extraction, filtration, precipitation, crystallization, or any other method known in the art. In certain embodiments, a compound or mixture is carried forward to the next synthetic step without purification (i.e., crude).

The synthetic method provided herein can be carried out on any scale (i.e., to yield any amount of product). In certain embodiments, the methods are applicable to small-scale synthesis or larger-scale process manufacture. In certain embodiments, a reaction provided herein is carried out to yield less than 1 g of product. In certain embodiments, a reaction provided herein is carried out to yield greater than 1 g, 2 g, 5 g, 10 g, 15 g, 20 g, 25 g, 30 g, 40 g, 50 g, 100 g, 200 g, 500 g, or 1 kg of product.

Methods for Delivery of Therapeutic Agents

Also provided herein are methods of delivering one or more therapeutic agents to a subject, the methods comprising administering to the subject an LNP described herein, wherein the LNP encapsulates the one or more therapeutic agents of interest.

In certain embodiments, the LNPs described herein may be less susceptible to clearance and thus have a longer half-life *in vivo*. This is particularly the case where the

compositions are intended for repeated, including chronic, administration, and even more particularly where such repeated administration occurs within days or weeks. Significantly, these compositions are less susceptible or altogether circumvent the observed phenomenon of accelerated blood clearance (ABC). ABC is a phenomenon in which certain exogenously administered agents are rapidly cleared from the blood upon second and subsequent administrations. This phenomenon has been observed, in part, for a variety of lipid-containing compositions including, but not limited to, lipidated agents, liposomes, or other lipid-based delivery vehicles, and lipid-encapsulated agents. This resistance to ABC renders these compounds and compositions particularly suitable for repeated use *in vivo*, including for repeated use within short periods of time, including days or 1-2 weeks. This enhanced stability and/or half-life is due, in part, to the inability of these compositions to activate B1a and/or B1b cells and/or conventional B cells, pDCs, and/or platelets. B1a cells are normally responsible for secreting natural antibody, in the form of circulating IgM. The IgM is poly-reactive, meaning that it is able to bind to a variety of antigens, albeit with a relatively low affinity for each.

It has been found that some lipidated agents or lipid-comprising formulations such as lipid nanoparticles administered *in vivo* trigger and are subject to ABC. It has also been found that upon administration of a first dose of the LNP, one or more cells involved in generating an innate immune response (referred to herein as sensors) bind such agent, are activated, and then initiate a cascade of immune factors (referred to herein as effectors) that promote ABC and toxicity. For instance, B1a and B1b cells may bind to LNP, become activated (alone or in the presence of other sensors such as pDC and/or effectors such as IL6) and secrete natural IgM that binds to the LNP. Pre-existing natural IgM in the subject may also recognize and bind to the LNP, thereby triggering complement fixation. After administration of the first dose, the production of natural IgM begins within 1-2 hours of administration of the LNP. Typically by about 2-3 weeks the natural IgM is cleared from the system due to the natural half-life of IgM. Natural IgG is produced beginning around 96 hours after administration of the LNP. The agent, when administered in a naïve setting, can exert its biological effects relatively unencumbered by the natural IgM produced post-activation of the B1a cells or B1b cells or natural IgG. The natural IgM and natural IgG are non-specific and thus are distinct from anti-PEG IgM and anti-PEG IgG.

For further details of the mechanisms associated with ABC, see International Publication Number WO 2017/099823, published Jun. 15, 2017, the entire contents of which is incorporated herein by reference.

In certain embodiments, an LNP described herein (i.e., comprising a plurality of PEG lipids at a specified purity described herein) is less sensitive to accelerated blood clearance (ABC) as compared to an LNP comprising a plurality of PEG lipids with a purity of less than 87%. For example, in certain embodiments, an LNP formulation comprising a plurality of compounds of Formula (I) as described herein is less sensitive to ABC as compared to an LNP formulation comprising a plurality of compounds of Formula (I) with a purity of less than 87%.

As described herein, this disclosure provides compounds and compositions that do not promote ABC. These may be further characterized as not capable of activating B1a and/or B1b cells, platelets, and/or pDC, and optionally conventional B cells also. These lipid nanoparticles are particularly desirable for applications requiring repeated administration,

and in particular repeated administrations that occur within with short periods of time (e.g., within 1-2 weeks). This is the case, for example, if the agent is a nucleic acid based therapeutic that is provided to a subject at regular, closely-spaced intervals.

The LNPs provided herein, in some instances, may not trigger a CD5(+) mediated immune response. As used herein, a CD5(+) mediated immune response is an immune response that is mediated by B1a and/or B1b cells. Such a response may include an ABC response, an acute phase response, induction of natural IgM and/or IgG, and the like.

The LNPs provided herein, in some instances, may not trigger a CD19(+) mediated immune response. As used herein, a CD19(+) mediated immune response is an immune response that is mediated by conventional CD19(+), CD5(-) B cells. Such a response may include induction of IgM, induction of IgG, induction of memory B cells, an ABC response, an anti-drug antibody (ADA) response including an anti-protein response where the protein may be encapsulated within an LNP, and the like.

B1a cells are a subset of B cells involved in innate immunity. These cells are the source of circulating IgM, referred to as natural antibody or natural serum antibody. Natural IgM antibodies are characterized as having weak affinity for a number of antigens, and therefore they are referred to as "poly-specific" or "poly-reactive," indicating their ability to bind to more than one antigen. B1a cells are not able to produce IgG. Additionally, they do not develop into memory cells and thus do not contribute to an adaptive immune response. However, they are able to secrete IgM upon activation. The secreted IgM is typically cleared within about 2-3 weeks, at which point the immune system is rendered relatively naïve to the previously administered antigen. If the same antigen is presented after this time period (e.g., at about 3 weeks after the initial exposure), the antigen is not rapidly cleared. However, significantly, if the antigen is presented within that time period (e.g., within 2 weeks, including within 1 week, or within days), then the antigen is rapidly cleared. This delay between consecutive doses has rendered certain lipid-containing therapeutic or diagnostic agents unsuitable for use.

In humans, B1a cells are CD19(+), CD20(+), CD27(+), CD43(+), CD70(-) and CD5(+). In mice, B1a cells are CD19(+), CD5(+), and CD45 B cell isoform B220(+). It is the expression of CD5 which typically distinguishes B1a cells from other convention B cells. B1a cells may express high levels of CD5, and on this basis may be distinguished from other B-1 cells such as B-1b cells which express low or undetectable levels of CD5. CD5 is a pan-T cell surface glycoprotein. B1a cells also express CD36, also known as fatty acid translocase. CD36 is a member of the class B scavenger receptor family. CD36 can bind many ligands, including oxidized low density lipoproteins, native lipoproteins, oxidized phospholipids, and long-chain fatty acids.

B1b cells are another subset of B cells involved in innate immunity. These cells are another source of circulating natural IgM. Several antigens, including PS, are capable of inducing T cell independent immunity through B1b activation. CD27 is typically upregulated on B1b cells in response to antigen activation. Similar to B1a cells, the B1b cells are typically located in specific body locations such as the spleen and peritoneal cavity and are in very low abundance in the blood. The B1b secreted natural IgM is typically cleared within about 2-3 weeks, at which point the immune system is rendered relatively naïve to the previously administered antigen. If the same antigen is presented after this time period (e.g., at about 3 weeks after the initial exposure),

the antigen is not rapidly cleared. However, significantly, if the antigen is presented within that time period (e.g., within 2 weeks, including within 1 week, or within days), then the antigen is rapidly cleared. This delay between consecutive doses has rendered certain lipid-containing therapeutic or diagnostic agents unsuitable for use.

In some embodiments it is desirable to block B1a and/or B1b cell activation. One strategy for blocking B1a and/or B1b cell activation involves determining which components of a lipid nanoparticle promote B cell activation and neutralizing those components. It has been discovered herein that at least PEG and phosphatidylcholine (PC) contribute to B1a and B1b cell interaction with other cells and/or activation. PEG may play a role in promoting aggregation between B1 cells and platelets, which may lead to activation. PC (a helper lipid in LNPs) is also involved in activating the B1 cells, likely through interaction with the CD36 receptor on the B cell surface. Numerous particles having PEG-lipid alternatives, PEG-less, and/or PC replacement lipids (e.g. oleic acid or analogs thereof) have been designed and tested. As described herein, utilizing PEG lipids of a recommended purity provided herein is useful in preventing ABC by reducing the production of natural IgM and/or B cell activation by LNPs. Thus, the invention encompasses LNPs that have reduced ABC as a result of a design which eliminates B cell triggers.

Another strategy for blocking B1a and/or B1b cell activation involves using an agent that inhibits immune responses induced by LNPs. These types of agents are discussed in more detail below. In some embodiments these agents block the interaction between B1a/B1b cells and the LNP or platelets or pDC. For instance the agent may be an antibody or other binding agent that physically blocks the interaction. An example of this is an antibody that binds to CD36 or CD6. The agent may also be a compound that prevents or disables the B1a/B1b cell from signaling once activated or prior to activation. For instance, it is possible to block one or more components in the B1a/B1b signaling cascade the results from B cell interaction with LNP or other immune cells. In other embodiments the agent may act one or more effectors produced by the B1a/B1b cells following activation. These effectors include for instance, natural IgM and cytokines.

In certain embodiments, a LNP comprising a plurality of PEG lipids described herein exhibits lower B cell (e.g., B1a, B1b cell) binding and/or activation as compared to an LNP formulation comprising a plurality of PEG lipids with a purity of less than 87%. For example, in certain embodiments, a LNP comprising a plurality of compounds of Formula (I) described herein exhibits lower B cell (e.g., B1a, B1b cell) binding and/or activation binding as compared to an LNP formulation comprising a plurality of compounds of Formula (I) with a purity of less than 87%.

In certain embodiments, a LNP comprising a plurality of PEG lipids described herein exhibits lower IgM binding as compared to an LNP formulation comprising a plurality of PEG lipids with a purity of less than 87%. For example, in certain embodiments, a LNP comprising a plurality of compounds of Formula (I) described herein exhibits lower IgM binding as compared to an LNP formulation comprising a plurality of compounds of Formula (I) with a purity of less than 87%.

In some instances, a method for delivering one or more therapeutic agents may comprise:

- (i) administering a first dose of an agent to a subject,
- (ii) administering a second or subsequent dose of the agent to the subject, wherein the second or subsequent dose is administered within 2 weeks of the first or prior dose, and
- (iii) repeating step (ii) one or more times, wherein the agent is formulated with an LNP that does not promote ABC.

10 Another method for delivering an agent to a subject involves

- (i) administering a first dose of an agent to a subject,
- (ii) administering a second or subsequent dose of the agent to the subject, wherein the second or subsequent dose is administered within 2 weeks of the first or prior dose, and
- (iii) repeating step (ii) one or more times,

15 wherein the half-life of the agent after the second and subsequent dose is at least 50%, 60%, 70%, 80%, 85%, 90%, 20 95% or more of the half-life of the agent after the first dose.

Still another method for delivering an agent to a subject involves:

- (i) administering a first dose of an agent to a subject,
- (ii) administering a second or subsequent dose of the agent to the subject, wherein the second or subsequent dose is administered within 2 weeks of the first or prior dose, and
- (iii) repeating step (ii) one or more times,

25 wherein the activity or blood concentration of the agent after the second and subsequent dose is at least 50%, 60%, 70%, 30 80%, 85%, 90%, 95% or more of the activity or blood concentration of the agent after the first dose.

Second or subsequent doses may be administered within 1 week, or within 6 days, or within 5 days, or within 4 days, or within 3 days, or within 2 days, or within 1 day of the first or prior dose. Agents may be administered two or more times, three or more times, four or more times, etc. Agent administration may therefore be repeated once, twice, 3, 4, 5, 6, 7, 8, 9, 10, or more times. The agent may be administered chronically or acutely, depending on its intended purpose.

40 The second and subsequent doses of biologically active agent may maintain an activity of at least 50% of the activity of the first dose, or at least 60% of the first dose, or at least 70% of the first dose, or at least 75% of the first dose, or at least 80% of the first dose, or at least 85% of the first dose, or at least 90% of the first dose, or at least 95% of the first dose, or more, for at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days post-administration of the second or subsequent dose.

The interval between the first dose and the second dose in 50 any of the methods described herein may be equal to or less than two weeks, for example, less than 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 days. In some instances, the subject can be administered a dose once daily, every 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 55 19, 20, or 21 days in any of the methods described herein. Each possibility represents a separate embodiment of the present invention.

When the biologically active agent is an mRNA (a therapeutic mRNA or a mRNA encoding a vaccine antigen), a 60 method for reducing ABC of LNPs encapsulating the mRNA can be performed using a low amount of the LNPs for the first dose, and/or the second dose (as well as the subsequent doses). The low doses can be equal to or less than 0.3 mg/kg, e.g., 0.2 mg/kg, or 0.1 mg/kg. In some instances, the first dose, the second dose, or both range from 0.1 to 0.3 mg/kg.

65 Also provided herein are methods of treating a subject having or at risk of having a condition that benefits from the

biologically active agent, particularly if the biologically active agent is a therapeutic agent. Alternatively, a method may be a method of diagnosing a subject, in which case the biologically active agent is a diagnostic agent.

LNP Formulations

The formation of a lipid nanoparticle (LNP) described herein may be accomplished by any methods known in the art. For example, as described in U.S. Pub. No. 2012/0178702, incorporated herein by reference in its entirety. Non-limiting examples of lipid nanoparticle compositions and methods of making them are described, for example, in Semple et al. (2010) *Nat. Biotechnol.* 28:172-176; Jayarama et al. (2012), *Angew. Chem. Int. Ed.*, 51: 8529-8533; and Maier et al. (2013) *Molecular Therapy* 21, 1570-1578 (the contents of each of which are incorporated herein by reference in their entirety).

In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO 2011/127255 or WO 2008/103276, the contents of each of which is herein incorporated by reference in their entirety. As a non-limiting example, LNP formulations as described in WO 2011/127255 and/or WO 2008/103276; each of which is herein incorporated by reference in their entirety. In one embodiment, LNP formulations described herein may comprise a polycationic composition. As a non-limiting example, the polycationic composition may be selected from formula 1-60 of US Patent Publication No. US 20050222064; the content of which is herein incorporated by reference in its entirety.

In one embodiment, the lipid nanoparticle may be formulated by the methods described in US Patent Publication No US 2013/0156845, and International Publication No WO 2013/093648 or WO 2012/024526, each of which is herein incorporated by reference in its entirety.

The lipid nanoparticles described herein may be made in a sterile environment by the system and/or methods described in US Patent Publication No. US 20130164400, which is incorporated herein by reference in its entirety.

In one embodiment, the LNP formulation may be formulated in a nanoparticle such as a nucleic acid-lipid particle described in U.S. Pat. No. 8,492,359, which is incorporated herein by reference in its entirety.

A nanoparticle composition may optionally comprise one or more coatings. For example, a nanoparticle composition may be formulated in a capsule, film, or tablet having a coating. A capsule, film, or tablet including a composition described herein may have any useful size, tensile strength, hardness, or density.

In some embodiments, such LNPs are synthesized using methods comprising microfluidic mixers. Exemplary microfluidic mixers may include, but are not limited to a slit interdigital micromixer including, but not limited to those manufactured by Microinnova (Allerheiligen bei Wildon, Austria) and/or a staggered herringbone micromixer (SHM) (Zhigaltsev, I. V. et al., Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing have been published (Langmuir. 2012. 28:3633-40; Belliveau, N. M. et al., Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. Molecular Therapy-Nucleic Acids. 2012. 1:e37; Chen, D. et al., Rapid discovery of potent siRNA-containing lipid nanoparticles enabled by controlled microfluidic formulation. *J Am Chem Soc.* 2012. 134(16):6948-51; each of which is herein incorporated by reference in its entirety).

In some embodiments, methods of LNP generation comprising SHM, further comprise the mixing of at least two

input streams wherein mixing occurs by microstructure-induced chaotic advection (MICA). According to this method, fluid streams flow through channels present in a herringbone pattern causing rotational flow and folding the fluids around each other. This method may also comprise a surface for fluid mixing wherein the surface changes orientations during fluid cycling. Methods of generating LNPs using SHM include those disclosed in U.S. Application Publication Nos. 2004/0262223 and 2012/0276209, each of which is incorporated herein by reference in their entirety.

In one embodiment, the lipid nanoparticles may be formulated using a micromixer such as, but not limited to, a Slit Interdigital Microstructured Mixer (SIMM-V2) or a Standard Slit Interdigital Micro Mixer (SSIMM) or Caterpillar (CPMM) or Impinging-jet (IJMM) from the Institut für Mikrotechnik Mainz GmbH, Mainz Germany.

In one embodiment, the lipid nanoparticles are created using microfluidic technology (see Whitesides, George M. The Origins and the Future of Microfluidics. *Nature*, 2006 442: 368-373; and Abraham et al. Chaotic Mixer for Micro-channels. *Science*, 2002 295: 647-651; each of which is herein incorporated by reference in its entirety). As a non-limiting example, controlled microfluidic formulation includes a passive method for mixing streams of steady pressure-driven flows in micro channels at a low Reynolds number (See e.g., Abraham et al. Chaotic Mixer for Micro-channels. *Science*, 2002 295: 647651; which is herein incorporated by reference in its entirety).

In one embodiment, the mRNA of the present invention may be formulated in lipid nanoparticles created using a micromixer chip such as, but not limited to, those from Harvard Apparatus (Holliston, MA) or Dolomite Microfluidics (Royston, UK). A micromixer chip can be used for rapid mixing of two or more fluid streams with a split and recombine mechanism.

In one embodiment, the lipid nanoparticles may have a diameter from about 10 to about 100 nm such as, but not limited to, about 10 to about 20 nm, about 10 to about 30 nm, about 10 to about 40 nm, about 10 to about 50 nm, about 10 to about 60 nm, about 10 to about 70 nm, about 10 to about 80 nm, about 10 to about 90 nm, about 20 to about 30 nm, about 20 to about 40 nm, about 20 to about 50 nm, about 20 to about 60 nm, about 20 to about 70 nm, about 20 to about 80 nm, about 20 to about 90 nm, about 20 to about 100 nm, about 30 to about 40 nm, about 30 to about 50 nm, about 30 to about 60 nm, about 30 to about 70 nm, about 30 to about 80 nm, about 30 to about 90 nm, about 30 to about 100 nm, about 40 to about 50 nm, about 40 to about 60 nm, about 40 to about 70 nm, about 40 to about 80 nm, about 40 to about 90 nm, about 40 to about 100 nm, about 50 to about 60 nm, about 50 to about 70 nm, about 50 to about 80 nm, about 50 to about 90 nm, about 50 to about 100 nm, about 60 to about 70 nm, about 60 to about 80 nm, about 60 to about 90 nm, about 60 to about 100 nm, about 70 to about 80 nm, about 70 to about 90 nm, about 70 to about 100 nm, about 80 to about 90 nm, about 80 to about 100 nm and/or about 90 to about 100 nm. In one embodiment, the lipid nanoparticles may have a diameter from about 10 to 500 nm. In one embodiment, the lipid nanoparticle may have a diameter greater than 100 nm, greater than 150 nm, greater than 200 nm, greater than 250 nm, greater than 300 nm, greater than 350 nm, greater than 400 nm, greater than 450 nm, greater than 500 nm, greater than 550 nm, greater than 600 nm, greater than 650 nm, greater than 700 nm, greater than 750 nm, greater than 800 nm, greater than 850 nm, greater than

900 nm, greater than 950 nm or greater than 1000 nm. Each possibility represents a separate embodiment of the present invention.

In some embodiments, a nanoparticle (e.g., a lipid nanoparticle) has a mean diameter of 10-500 nm, 20-400 nm, 30-300 nm, or 40-200 nm. In some embodiments, a nanoparticle (e.g., a lipid nanoparticle) has a mean diameter of 50-150 nm, 50-200 nm, 80-100 nm, or 80-200 nm.

In some embodiments, the lipid nanoparticles described herein can have a diameter from below 0.1 um up to 100 nm such as, but not limited to, less than 0.1 um, less than 1.0 um, less than 5 um, less than 10 um, less than 15 um, less than 20 um, less than 25 um, less than 30 um, less than 35 um, less than 40 um, less than 50 um, less than 55 um, less than 60 um, less than 65 um, less than 70 um, less than 75 um, less than 80 um, less than 85 um, less than 90 um, less than 95 um, less than 100 um, less than 125 um, less than 150 um, less than 175 um, less than 200 um, less than 225 um, less than 250 um, less than 275 um, less than 300 um, less than 325 um, less than 350 um, less than 375 um, less than 400 um, less than 425 um, less than 450 um, less than 475 um, less than 500 um, less than 525 um, less than 550 um, less than 575 um, less than 600 um, less than 625 um, less than 650 um, less than 675 um, less than 700 um, less than 725 um, less than 750 um, less than 775 um, less than 800 um, less than 825 um, less than 850 um, less than 875 um, less than 900 um, less than 925 um, less than 950 um, less than 975 um.

In another embodiment, LNPs may have a diameter from about 1 nm to about 100 nm, from about 1 nm to about 10 nm, about 1 nm to about 20 nm, from about 1 nm to about 30 nm, from about 1 nm to about 40 nm, from about 1 nm to about 50 nm, from about 1 nm to about 60 nm, from about 1 nm to about 70 nm, from about 1 nm to about 80 nm, from about 1 nm to about 90 nm, from about 5 nm to about 100 nm, from about 5 nm to about 10 nm, about 5 nm to about 20 nm, from about 5 nm to about 30 nm, from about 5 nm to about 40 nm, from about 5 nm to about 50 nm, from about 5 nm to about 60 nm, from about 5 nm to about 70 nm, from about 5 nm to about 80 nm, from about 5 nm to about 90 nm, about 10 to about 50 nM, from about 20 to about 50 nm, from about 30 to about 50 nm, from about 40 to about 50 nm, from about 20 to about 60 nm, from about 30 to about 60 nm, from about 40 to about 60 nm, from about 20 to about 70 nm, from about 30 to about 70 nm, from about 40 to about 70 nm, from about 50 to about 70 nm, from about 60 to about 70 nm, from about 20 to about 80 nm, from about 30 to about 80 nm, from about 40 to about 80 nm, from about 50 to about 80 nm, from about 20 to about 90 nm, from about 30 to about 90 nm, from about 40 to about 90 nm, from about 50 to about 90 nm, from about 60 to about 90 nm and/or from about 70 to about 90 nm. Each possibility represents a separate embodiment of the present invention.

A nanoparticle composition may be relatively homogenous. A polydispersity index may be used to indicate the homogeneity of a nanoparticle composition, e.g., the particle size distribution of the nanoparticle compositions. A small (e.g., less than 0.3) polydispersity index generally indicates a narrow particle size distribution. A nanoparticle composition may have a polydispersity index from about 0 to about 0.25, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, or 0.25. In some embodiments, the polydispersity index of a nanoparticle composition may be from about 0.10 to about 0.20. Each possibility represents a separate embodiment of the present invention.

The zeta potential of a nanoparticle composition may be used to indicate the electrokinetic potential of the composition. For example, the zeta potential may describe the surface charge of a nanoparticle composition. Nanoparticle compositions with relatively low charges, positive or negative, are generally desirable, as more highly charged species may interact undesirably with cells, tissues, and other elements in the body. In some embodiments, the zeta potential of a nanoparticle composition may be from about -10 mV to about +20 mV, from about -10 mV to about +10 mV, from about -10 mV to about +5 mV, from about -10 mV to about 0 mV, from about -10 mV to about -5 mV, from about -5 mV to about +20 mV, from about -5 mV to about +15 mV, from about -5 mV to about +10 mV, from about -5 mV to about +5 mV, from about -5 mV to about 0 mV, from about 0 mV to about +20 mV, from about 0 mV to about +15 mV, from about 0 mV to about +10 mV, from about 0 mV to about +5 mV, from about +5 mV to about +20 mV, from about +5 mV to about +15 mV, or from about +5 mV to about +10 mV. Each possibility represents a separate embodiment of the present invention.

The efficiency of encapsulation of a therapeutic agent describes the amount of therapeutic agent that is encapsulated or otherwise associated with a nanoparticle composition after preparation, relative to the initial amount provided. The encapsulation efficiency is desirably high (e.g., close to 100%). The encapsulation efficiency may be measured, for example, by comparing the amount of therapeutic agent in a solution containing the nanoparticle composition before and after breaking up the nanoparticle composition with one or more organic solvents or detergents. Fluorescence may be used to measure the amount of free therapeutic agent (e.g., nucleic acids) in a solution. For the nanoparticle compositions described herein, the encapsulation efficiency of a therapeutic agent may be at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the encapsulation efficiency may be at least 80%. In certain embodiments, the encapsulation efficiency may be at least 90%. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the lipid nanoparticle has a polydispersity value of less than 0.4. In some embodiments, the lipid nanoparticle has a net neutral charge at a neutral pH. In some embodiments, the lipid nanoparticle has a mean diameter of 50-200 nm.

The properties of a lipid nanoparticle formulation may be influenced by factors including, but not limited to, the selection of the cationic lipid component, the degree of cationic lipid saturation, the selection of the non-cationic lipid component, the degree of non-cationic lipid saturation, the selection of the structural lipid component, the nature of the PEGylation, ratio of all components and biophysical parameters such as size. As described herein, the purity of a PEG lipid component is also important to an LNP's properties and performance.

In certain embodiments, when a lipid nanoparticle (LNP) comprises phospholipids and encapsulates an oligonucleotide, polynucleotide, or nucleic acid, the LNP has an N/P ratio. "N/P ratio" refers to the ratio of positively-charged polymer amines (e.g., nitrogens from phosphatidylcholine groups on phospholipids) in the nanoparticle ("N") to phosphate groups in the oligonucleotide, polynucleotide, or nucleic acid agent ("P"). The N/P ratio of a LNP can influence many properties such as its net surface charge, size, stability, and in vivo activity. In certain embodiments,

an LNP described herein has an N/P ratio from 1.0 to 10.0, inclusive. In certain embodiments, an LNP described herein has an N/P ratio of approximately 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10.0. In certain embodiments, the N/P ratio is from 1.0 to 5.0, inclusive. In certain embodiments, the N/P ratio is from 2.0 to 5.0, inclusive. In certain embodiments, the N/P ratio is from 2.0 to 4.0, inclusive. In certain embodiments, the N/P ratio is approximately 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, or 4.0. In certain embodiments, the N/P ratio is from 2.5 to 3.5, inclusive. In certain embodiments, the N/P ratio is approximately 2.0. In certain embodiments, the N/P ratio is approximately 3.0. In certain embodiments, the N/P ratio is approximately 4.0. In certain embodiments, the N/P ratio is approximately 5.0.

Agents and Therapeutic Agents

This disclosure contemplates that the LNPs provided herein and/or the various combination therapies provided herein may be used to deliver a variety of agents to a subject. Such agents typically will be biologically active agents. Biologically active agents are agents that have an effect *in vivo*, and preferably a beneficial effect, such as desirable immune modulation, immune stimulation, immune inhibition, cell killing, cell preservation, modified gene expression, protein replacement, and the like. Biologically active agents include but are not limited to prophylactic agents, therapeutic agents, and diagnostic agents. Biologically active agents include immunomodulatory agents such as immunostimulatory or immunoinhibitory agents, antigens, antibodies and antibody fragments such as antigen-binding antibody fragments, adjuvants, cytokines such as interleukins, anti-bacterial agents, anti-viral agents, anti-fungal agents, anti-parasitic agents, anti-cancer agents, anti-inflammatory agents, and the like.

Such agents may be, without limitation, nucleic acids, proteins or peptide, small organic compounds, carbohydrates and/or polysaccharides, and the like. They may be used to express nucleic acids and/or proteins in cells, particularly in cells that are deficient in such nucleic acids or proteins or have mutated versions of such nucleic acids or proteins. They may be used to introduce and express nucleic acids or proteins that are not native to the cell or organism, as may be done for example in the context of an immunization or vaccination protocol. In this respect, the nucleic acid or protein may be foreign to the subject to whom it is administered (e.g., not naturally occurring in such subject, or not naturally occurring at all), and it is administered to the subject to induce and/or boost an immune response to such nucleic acid or protein. The nucleic acids provided herein may be used for such a purpose.

Other biologically active agents may be used alone or together with such nucleic acids or proteins, including formulated together with such nucleic acids or proteins, including formulated in the LNPs of this disclosure.

As used herein, the term "nucleic acid" refers to a compound comprising a nucleobase and an acidic moiety, e.g., a nucleoside, a nucleotide, or a polymer of nucleotides. Typically, polymeric nucleic acids, e.g., nucleic acid molecules comprising three or more nucleotides are linear molecules, in which adjacent nucleotides are linked to each other via a phosphodiester linkage. In some embodiments, "nucleic acid" refers to individual nucleic acid residues (e.g., nucleotides and/or nucleosides). In some embodiments, "nucleic acid" refers to an oligonucleotide chain comprising three or more individual nucleotide residues. As used herein, the terms "oligonucleotide" and "polynucleotide" can be

used interchangeably to refer to a polymer of nucleotides (e.g., a string of at least three nucleotides). In some embodiments, "nucleic acid" encompasses RNA as well as single and/or double-stranded DNA. A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated.

Nucleic acids include any compound and/or substance that comprises a polymer of nucleotides. These polymers are referred to as polynucleotides. Nucleic acids may be or may include, for example, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β -D-ribo configuration, α -LNA having an α -L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino- α -LNA having a 2'-amino functionalization), ethylene nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or chimeras or combinations thereof.

Nucleic acids may be naturally occurring, for example, in the context of a genome, a transcript, an mRNA, tRNA, rRNA, siRNA, snRNA, plasmid, cosmid, chromosome, chromatid, or other naturally occurring nucleic acid molecule. On the other hand, a nucleic acid molecule may be a non-naturally occurring molecule, e.g., a recombinant DNA or RNA, an artificial chromosome, an engineered genome, or fragment thereof, or a synthetic DNA, RNA, DNA/RNA hybrid, or including non-naturally occurring nucleotides or nucleosides. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, etc.

Furthermore, the terms "nucleic acid," "DNA," "RNA," and/or similar terms include nucleic acid analogs, i.e., analogs having other than a phosphodiester backbone.

Where appropriate, e.g., in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, and backbone modifications. In some embodiments, a nucleic acid is or comprises natural nucleosides (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyluridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

A "nucleoside" refers to a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). A "nucleotide" refers to a nucleoside, including a phosphate group. Modified nucleotides may be synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Polynucleotides may comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages may be standard phosphodiester linkages, in which case the polynucleotides would comprise regions of nucleotides.

Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil. Any combination of base/sugar or linker may be incorporated into polynucleotides of the present disclosure.

The skilled artisan will appreciate that, except where otherwise noted, polynucleotide sequences set forth in the instant application will recite "T"s in a representative DNA sequence but where the sequence represents RNA, the "T"s would be substituted for "U"s.

Modifications of polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) that are useful in the therapeutic agents described herein include, but are not limited to the following: 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine; 2-methylthio-N6-methyladenosine; 2-methylthio-N6-threonyl carbamoyladenidine; N6-glycylcarbamoyladenidine; N6-isopentenyladenidine; N6-methyladenidine; N6-threonylcarbamoyladenidine; 1,2'-O-dimethyladenidine; 1-methyladenidine; 2'-O-methyladenidine; 2'-O-ribosyladenidine (phosphate); 2-methyladenidine; 2-methylthio-N6 isopentenyladenidine; 2-methylthio-N6-hydroxynorvalyl carbamoyladenidine; 2'-O-methyladenidine; 2'-O-ribosyladenidine (phosphate); Isopentenyladenidine; N6-(cis-hydroxyisopentenyl)adenidine; N6,2'-O-dimethyladenidine; N6,2'-O-dimethyladenidine; N6,N6,2'-O-trimethyladenidine; N6,N6-dimethyladenidine; N6-acetyladenidine; N6-hydroxynorvalylcarbamoyladenidine; N6-methyl-N6-threonylcarbamoyladenidine; 2-methyladenidine; 2-methylthio-N6-isopentenyladenidine; 7-deaza-adenidine; N1-methyl-adenidine; N6, N6 (dimethyl)adenine; N6-cis-hydroxy-isopentenyladenidine; α -thio-adenidine; 2 (amino)adenine; 2 (aminopropyl)adenine; 2 (methylthio) N6 (isopentenyl)adenine; 2-(alkyl)adenine; 2-(aminoalkyl)adenine; 2-(aminopropyl)adenine; 2-(halo)adenine; 2-(halo)adenine; 2-(propyl)adenine; 2'-Amino-2'-deoxy-ATP; 2'-Azido-2'-deoxy-ATP; 2'-Deoxy-2'-a-aminoadenidine TP; 2'-Deoxy-2'-a-azidoadenidine TP; 6 (alkyl)adenine; 6 (methyl)adenine; 6-(alkyl)adenine; 6-(methyl)adenine; 7 (deaza)adenine; 8 (alkenyl)adenine; 8 (alkynyl)adenine; 8 (amino)adenine; 8 (thioalkyl)adenine; 8-(alkenyl)adenine; 8-(alkyl)adenine; 8-(alkynyl)adenine; 8-(amino)adenine; 8-(halo)adenine; 8-(hydroxyl)adenine; 8-(thioalkyl)adenine; 8-(thiol)adenine; 8-azido-adenidine; aza adenine; deaza adenine; N6 (methyl)adenine; N6-(isopentyl)adenine; 7-deaza-8-aza-adenidine; 7-methyladenidine; 1-Deazaadenidine TP; 2'Fluoro-N6-Bz-deoxyadenosine TP; 2'-OMe-2-Amino-ATP; 2'O-methyl-N6-Bz-deoxyadenosine TP; 2'-a-Ethyanyladenosine TP; 2-aminoadenine; 2-Aminoadenosine TP; 2-Amino-ATP; 2'-a-Trifluoromethyladenosine TP; 2-Azidoadenosine TP; 2'-b-Ethyanyladenosine TP; 2-Bromoadenosine TP; 2'-b-Trifluoromethyladenosine TP; 2-Chloroadenosine TP; 2'-Deoxy-2',2'-difluoroadenosine TP; 2'-Deoxy-2'-a-mercaptopadenosine TP; 2'-Deoxy-2'-a-thiomethoxyadenosine TP; 2'-Deoxy-2'-b-aminoadenosine TP; 2'-Deoxy-2'-b-azidoadenosine TP; 2'-Deoxy-2'-b-bromoadenosine TP; 2'-Deoxy-2'-b-chloroadenosine TP; 2'-Deoxy-2'-b-fluoroadenosine TP; 2'-Deoxy-2'-b-iodoadenosine TP; 2'-Deoxy-2'-b-mercaptopadenosine TP; 2'-Deoxy-2'-b-mercaptopadenosine TP; 2'-Deoxy-2'-b-

thiomethoxyadenosine TP; 2-Fluoroadenosine TP; 2-Iodo-adenosine TP; 2-Mercaptoadenosine TP; 2-methoxy-adenine; 2-methylthio-adenine; 2-Trifluoromethyladenosine TP; 3-Deaza-3-bromoadenosine TP; 3-Deaza-3-chloroadenosine TP; 3-Deaza-3-fluoroadenosine TP; 3-Deaza-3-iodoadenosine TP; 3-Deazaadenosine TP; 4'-Azidoadenosine TP; 4'-Carbocyclic adenosine TP; 4'-Ethyanyladenosine TP; 5'-Homo-adenosine TP; 8-Aza-ATP; 8-bromo-adenosine TP; 8-Trifluoromethyladenosine TP; 9-Deazaadenosine TP; 10 2-aminopurine; 7-deaza-2,6-diaminopurine; 7-deaza-8-aza-2,6-diaminopurine; 7-deaza-8-aza-2-aminopurine; 7-deaza-8-aza-adenine; 7-deaza-2-aminopurine; 2-thiocytidine; 3-methylcytidine; 5-formylcytidine; 5-hydroxymethylcytidine; 5-methylcytidine; N4-acetylcystidine; 2'-O-methylcytidine; 2'-O-methylcytidine; 5,2'-O-dimethylcytidine; 5-formyl-2'-O-methylcytidine; Lysidine; N4,2'-O-dimethylcytidine; N4-acetyl-2'-O-methylcytidine; N4-methylcytidine; N4,N4-Dimethyl-2'-OMe-Cytidine TP; 4-methylcytidine; 5-aza-cytidine; Pseudo-iso-cytidine; pyrrolo-cytidine; α -thio-cytidine; 2-(thio)cytosine; 2'-Amino-2'-deoxy-CTP; 2'-Azido-2'-deoxy-CTP; 2'-Deoxy-2'-a-aminocytidine TP; 2'-Deoxy-2'-a-azidocytidine TP; 3 (deaza) 5 (aza)cytosine; 3 (methyl)cytosine; 3-(alkyl)cytosine; 3-(deaza) 5 (aza)cytosine; 3-(methyl)cytosine; 4,2'-O-dimethylcytidine; 5 (halo)cytosine; 5 (methyl)cytosine; 5 (propynyl)cytosine; 5 (trifluoromethyl)cytosine; 5-(alkyl)cytosine; 5-(alkynyl)cytosine; 5-(halo)cytosine; 5-(propynyl) cytosine; 5-(trifluoromethyl)cytosine; 5-bromo-cytidine; 5-iodo-cytidine; 5-propynyl cytosine; 6-(azo)cytosine; 6-aza-cytidine; aza cytosine; deaza cytosine; N4 (acetyl) cytosine; 1-methyl-1-deaza-pseudoisocytidine; 1-methylpseudoisocytidine; 2-methoxy-5-methyl-cytidine; 2-methoxy-cytidine; 2-thio-5-methyl-cytidine; 4-methoxy-1-methyl-pseudoisocytidine; 4-methoxy-pseudoisocytidine; 4-thio-1-methyl-1-deaza-pseudoisocytidine; 4-thio-1-methyl-pseudoisocytidine; 4-thio-pseudoisocytidine; 5-azabularine; 5-methyl-zebularine; pyrrolo-pseudoisocytidine; Zebularine; (E)-5-(2-Bromo-vinyl)cytidine TP; 2,2'-anhydro-cytidine TP hydrochloride; 2'Fluor-N4-Bz-cytidine TP; 2'Fluoro-N4-Acetyl-cytidine TP; 2'-O-Methyl-N4-Acetyl-cytidine TP; 2'O-methyl-N4-Bz-cytidine TP; 2'-a-Ethyanylcytidine TP; 2'-a-Trifluoromethylcytidine TP; 2'-b-Ethyanylcytidine TP; 2'-b-Trifluoromethylcytidine TP; 2'-Deoxy-2',2'-difluorocytidine TP; 2'-Deoxy-2'-a-mercaptopcytidine TP; 2'-Deoxy-2'-a-thiomethoxyctidine TP; 2'-Deoxy-2'-b-aminocytidine TP; 2'-Deoxy-2'-b-bromocytidine TP; 2'-Deoxy-2'-b-chlorocytidine TP; 2'-Deoxy-2'-b-fluorocytidine TP; 2'-Deoxy-2'-b-iodocytidine TP; 2'-Deoxy-2'-b-mercaptopcytidine TP; 2'-Deoxy-2'-b-thiomethoxyctidine TP; 2'-O-Methyl-5-(1-propynyl)cytidine TP; 3'-Ethyanylcytidine TP; 4'-Azidocytidine TP; 4'-Carbocyclic cytidine TP; 4'-Ethyanylcytidine TP; 5-(1-Propynyl)ara-cytidine TP; 5-(2-Chloro-phenyl)-2-thiocytidine TP; 5-Aminoallyl-CTP; 5-Cyanocytidine TP; 5-Ethyanylara-cytidine TP; 5-Ethyanylcytidine TP; 5-Homo-cytidine TP; 5-Methoxycytidine TP; 5-Trifluoromethyl-Cytidine TP; N4-Amino-cytidine TP; N4-Benzoyl-cytidine TP; Pseudoisocytidine; 7-methylguanosine; N2,2'-O-dimethylguanosine; N2-methylguanosine; Wyosine; 1,2'-O-dimethylguanosine; 1-methylguanosine; 2'-O-methylguanosine; 2'-O-ribosylguanosine (phosphate); 2'-O-methylguanosine; 2'-O-ribosylguanosine (phosphate); 7-aminomethyl-7-deazaguanosine; 7-cyano-7-deazaguanosine; Archaeosine; Methylwyosine; N2,7-dimethylguanosine; N2,N2,2'-O-trimethylguanosine; N2,N2,7-trimethylguanosine; N2,N2-dimethylguanosine; N2,7,2'-O-trimethylguanosine; 6-thio-

guanosine; 7-deaza-guanosine; 8-oxo-guanosine; N1-methyl-guanosine; α -thio-guanosine; 2-(propyl)guanine; 2-(alkyl)guanine; 2'-Amino-2'-deoxy-GTP; 2'-Azido-2'-deoxy-GTP; 2'-Deoxy-2'-a-aminoguanosine TP; 2'-Deoxy-2'-a-azidoguanosine TP; 6 (methyl)guanine; 6-(alkyl)guanine; 6-(methyl)guanine; 6-methyl-guanosine; 7 (alkyl)guanine; 7 (deaza)guanine; 7 (methyl)guanine; 7-(alkyl)guanine; 7-(deaza)guanine; 7-(methyl)guanine; 8 (alkyl)guanine; 8 (alkynyl)guanine; 8 (halo)guanine; 8 (thioalkyl)guanine; 8-(alkenyl)guanine; 8-(alkyl)guanine; 8-(alkynyl)guanine; 8-(amino)guanine; 8-(halo)guanine; 8-(hydroxyl)guanine; 8-(thioalkyl)guanine; 8-(thiol)guanine; aza guanine; deaza guanine; N (methyl)guanine; N-(methyl)guanine; 1-methyl-6-thio-guanosine; 6-methoxy-guanosine; 6-thio-7-deaza-8-aza-guanosine; 6-thio-7-deaza-guanosine; 6-thio-7-methyl-guanosine; 7-deaza-8-aza-guanosine; 7-methyl-8-oxo-guanosine; N2,N2-dimethyl-6-thio-guanosine; N2-methyl-6-thio-guanosine; 1-Me-GTP; 2'Fluoro-N2-isobutyl-guanosine TP; 2'O-methyl-N2-isobutyl-guanosine TP; 2'-a-Ethynguanosine TP; 2'-a-Trifluoromethylguanosine TP; 2'-b-Ethynguanosine TP; 2'-b-Trifluoromethylguanosine TP; 2'-Deoxy-2',2'-difluoroguanosine TP; 2'-Deoxy-2'-a-mercaptopguanosine TP; 2'-Deoxy-2'-a-thiomethoxyguanosine TP; 2'-Deoxy-2'-b-aminoguanosine TP; 2'-Deoxy-2'-b-azidoguanosine TP; 2'-Deoxy-2'-b-bromoguanosine TP; 2'-Deoxy-2'-b-chloroguanosine TP; 2'-Deoxy-2'-b-fluoroguanosine TP; 2'-Deoxy-2'-b-iodoguanosine TP; 2'-Deoxy-2'-b-mercaptopguanosine TP; 2'-Deoxy-2'-b-thiomethoxyguanosine TP; 4'-Azidoguanosine TP; 4'-Carbocyclic guanosine TP; 4'-Ethynguanosine TP; 5'-Homo-guanosine TP; 8-bromo-guanosine TP; 9-Deazaguanosine TP; N2-isobutyl-guanosine TP; 1-methylinosine; Inosine; 1,2'-O-dimethylinosine; 2'-O-methylinosine; 7-methylinosine; 2'-O-methylinosine; Epoxyqueuosine; galactosyl-queuosine; Mannosylqueuosine; Queuosine; allyamino-thymidine; aza thymidine; deaza thymidine; deoxy-thymidine; 2'-O-methyluridine; 2-thiouridine; 3-methyluridine; 5-carboxymethyluridine; 5-hydroxyuridine; 5-methyluridine; 5-taurinomethyl-2-thiouridine; 5-taurinomethyluridine; Dihydrouridine; Pseudouridine; (3-(3-amino-3-carboxypropyl)uridine; 1-methyl-3-(3-amino-5-carboxypropyl)pseudouridine; 1-methylpsedouridine; 1-methyl-pseudouridine; 2'-O-methyluridine; 2'-O-methylpseudouridine; 2'-O-methyluridine; 2-thio-2'-O-methyluridine; 3-(3-amino-3-carboxypropyl)uridine; 3,2'-O-dimethyluridine; 3-Methyl-pseudo-Uridine TP; 4-thiouridine; 5-(carboxyhydroxymethyl)uridine; 5-(carboxyhydroxymethyl)uridine methyl ester; 5,2'-O-dimethyluridine; 5,6-dihydro-uridine; 5-aminomethyl-2-thiouridine; 5-carbamoylmethyl-2'-O-methyluridine; 5-carbamoylmethyluridine; 5-carboxyhydroxymethyluridine; 5-carboxyhydroxymethyluridine methyl ester; 5-carboxymethylaminomethyl-2'-O-methyluridine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyluridine; 5-carboxymethylaminomethyluridine; 5-Carbamoylmethyluridine TP; 5-methoxycarbonylmethyl-2'-O-methyluridine; 5-methoxycarbonylmethyl-2-thiouridine; 5-methoxycarbonylmethyluridine; 5-methyluridine; 5-methoxyuridine; 5-methyl-2-thiouridine; 5-methylaminomethyl-2-selenouridine; 5-methylaminomethyl-2-thiouridine; 5-methylaminomethyluridine; 5-Methylidihydrouridine; 5-Oxyacetic acid-Uridine TP; 5-Oxyacetic acid-methyl ester-Uridine TP; N1-methylpseudo-uridine; uridine 5-oxyacetic acid; uridine 5-oxyacetic acid methyl ester; 3-(3-Amino-3-carboxypropyl)-Uridine TP; 5-(iso-Pentenylaminomethyl)-2-thiouridine TP; 5-(iso-Pentenylaminomethyl)-2'-O-methyluridine TP;

5-(iso-Pentenylaminomethyl)uridine TP; 5-propynyl uracil; α -thio-uridine; 1 (aminoalkylamino-carbonylethylene)-2-(thio)pseudouracil; 1 (aminoalkylaminocarbonylethylene)-2,4-(dithio)pseudouracil; 1 (aminoalkylaminocarbonylethylene)-4 (thio)pseudouracil; 1 (aminoalkylaminocarbonylethylene)-pseudouracil; 1 (aminocarbonylethylene)-2,4-(dithio)pseudouracil; 1 (aminocarbonylethylene)-4 (thio)pseudouracil; 1 (aminocarbonylethylene)-pseudouracil; 1 substituted 2(thio)-pseudouracil; 1 substituted 2,4-(dithio)pseudouracil; 1 substituted 4 (thio)pseudouracil; 1 substituted pseudouracil; 1 1-(aminoalkylamino-carbonylethylene)-2-(thio)pseudouracil; 1-Methyl-3-(3-amino-3-carboxypropyl) pseudouridine TP; 1-Methyl-3-(3-amino-3-carboxypropyl) pseudo-UTP; 1-Methyl-pseudo-UTP; 2 (thio)pseudouracil; 2' deoxy uridine; 2' fluorouridine; 2-(thio)uracil; 2,4-(dithio) psuedouracil; 2' methyl, 2' amino, 2' azido, 2' fluro-guanosine; 2'-Amino-2'-deoxy-UTP; 2'-Azido-2'-deoxy-UTP; 2'-Azido-deoxyuridine TP; 2'-O-methylpseudouridine; 2' deoxy uridine; 2' fluorouridine; 2'-Deoxy-2'-a-aminouridine TP; 2'-Deoxy-2'-a-azidouridine TP; 2-methylpseudouridine; 3 (3 amino-3 carboxypropyl)uracil; 4 (thio)pseudouracil; 4-(thio)pseudouracil; 4-(thio)uracil; 4-thiouracil; 5 (1,3-diazole-1-alkyl)uracil; 5 (2-aminopropyl)uracil; 5 (aminoalkyl)uracil; 5 (dimethylaminoalkyl)uracil; 5 (guanidiniumalkyl)uracil; 5 (methoxycarbonylmethyl)-2-(thio)uracil; 5 (methoxycarbonyl-methyl)uracil; 5 (methyl) 2 (thio)uracil; 5 (methyl) 2,4 (dithio)uracil; 5 (methyl) 4 (thio)uracil; 5 (methylaminomethyl)-2 (thio)uracil; 5 (methylaminomethyl)-2,4 (dithio)uracil; 5 (methylaminomethyl)-4 (thio)uracil; 5 (propynyl)uracil; 5 (trifluoromethyl)uracil; 5-(2-aminopropyl)uracil; 5-(alkyl)-2-(thio)pseudouracil; 5-(alkyl)-2,4 (dithio)pseudouracil; 5-(alkyl)-4 (thio)pseudouracil; 5-(alkyl)pseudouracil; 5-(alkyl)uracil; 5-(alkynyl)uracil; 5-(allylamino)uracil; 5-(cyanoalkyl)uracil; 5-(dialkylaminoalkyl)uracil; 5-(dimethylaminoalkyl)uracil; 5-(guanidiniumalkyl)uracil; 5-(halo)uracil; 5-(1,3-diazole-1-alkyl)uracil; 5-(methoxy)uracil; 5-(methoxycarbonylmethyl)-2-(thio)uracil; 5-(methoxycarbonyl-methyl)uracil; 5-(methyl) 2 (thio)uracil; 5-(methyl) 2,4 (dithio)uracil; 5-(methyl) 4 (thio)pseudouracil; 5-(methylaminomethyl)-2 (thio)uracil; 5-(methylaminomethyl)-4-(thio)uracil; 5-(propynyl)uracil; 5-(trifluoromethyl)uracil; 5-aminoallyl-uridine; 5-bromo-uridine; 5-iodo-uridine; 5-uracil; 6 (azo)uracil; 6-(azo)uracil; 6-aza-uridine; allyamino-uracil; aza uracil; deaza uracil; N3 (methyl)uracil; Pseudo-UTP-1-2-ethanoic acid; Pseudouracil; 4-Thio-pseudo-UTP; 1-carboxymethyl-pseudouridine; 1-methyl-1-deaza-pseudouridine; 1-propynyl-uridine; 1-taurinomethyl-1-methyluridine; 1-taurinomethyl-4-thio-uridine; 1-taurinomethyl-pseudouridine; 2-methoxy-4-thio-pseudouridine; 2-thio-1-methyl-1-deaza-pseudouridine; 2-thio-1-methyl-pseudouridine; 2-thio-5-aza-uridine; 2-thio-dihydropseudouridine; 2-thio-dihydouridine; 2-thio-pseudouridine; 4-methoxy-2-thio-pseudouridine; 4-methoxy-pseudouridine; 4-thio-1-methyl-pseudouridine; 4-thio-pseudouridine; 5-aza-uridine; Dihydropseudouridine; (\pm)-1-(2-Hydroxypropyl)pseudouridine TP; (2R)-1-(2-Hydroxypropyl)pseudouridine TP; (2S)-1-(2-Hydroxypropyl)pseudouridine TP; (E)-5-(2-Bromo-vinyl)ara-uridine TP; (E)-5-(2-Bromo-vinyl)uridine TP; (Z)-5-(2-Bromo-vinyl)ara-uridine TP; (Z)-5-(2-Bromo-vinyl)uridine TP; 1-(2,2,2-Trifluoroethyl)-pseudo-UTP; 1-(2,2,3,3,3-Pentafluoropro-

pyl)pseudouridine TP; 1-(2,2-Diethoxyethyl)pseudouridine TP; 1-(2,4,6-Trimethylbenzyl)pseudouridine TP; 1-(2,4,6-Trimethyl-phenyl)pseudo-UTP; 1-(2-Amino-2-carboxyethyl)pseudo-UTP; 1-(2-Amino-ethyl)pseudo-UTP; 1-(2-Hydroxyethyl)pseudouridine TP; 1-(2-Methoxyethyl)pseudouridine TP; 1-(3,4-Bis-trifluoromethoxybenzyl)pseudouridine TP; 1-(3,4-Dimethoxybenzyl)pseudouridine TP; 1-(3-Amino-3-carboxypropyl)pseudo-UTP; 1-(3-Amino-propyl)pseudo-UTP; 1-(3-Cyclopropyl-prop-2-ynyl)pseudouridine TP; 1-(4-Amino-4-carboxybutyl)pseudo-UTP; 1-(4-Amino-benzyl)pseudo-UTP; 1-(4-Amino-butyl)pseudo-UTP; 1-(4-Amino-phenyl)pseudo-UTP; 1-(4-Azidobenzyl)pseudouridine TP; 1-(4-Bromobenzyl)pseudouridine TP; 1-(4-Chlorobenzyl)pseudouridine TP; 1-(4-Fluorobenzyl)pseudouridine TP; 1-(4-Iodobenzyl)pseudouridine TP; 1-(4-Methanesulfonylbenzyl)pseudouridine TP; 1-(4-Methoxybenzyl)pseudouridine TP; 1-(4-Methoxy-phenyl)pseudo-UTP; 1-(4-Methylbenzyl)pseudouridine TP; 1-(4-Methylbenzyl)pseudo-UTP; 1-(4-Nitrobenzyl)pseudouridine TP; 1-(4-Nitro-benzyl)pseudo-UTP; 1-(4-Nitro-phenyl)pseudo-UTP; 1-(4-Thiomethoxybenzyl)pseudouridine TP; 1-(4-Trifluoromethoxybenzyl)pseudouridine TP; 1-(4-Trifluoromethylbenzyl)pseudouridine TP; 1-(5-Amino-pentyl)pseudo-UTP; 1-(6-Amino-hexyl)pseudo-UTP; 1,6-Dimethyl-pseudo-UTP; 1-[3-{2-[2-(2-Aminoethoxy)-ethoxy]-ethoxy}-propionyl]pseudouridine TP; 1-{3-[2-(2-Aminoethoxy)-ethoxy]-propionyl} pseudouridine TP; 1-Acetyl pseudouridine TP; 1-Alkyl-6-(1-propynyl)-pseudo-UTP; 1-Alkyl-6-(2-propynyl)-pseudo-UTP; 1-Alkyl-6-allyl-pseudo-UTP; 1-Alkyl-6-ethynyl-pseudo-UTP; 1-Alkyl-6-homoallylpseudo-UTP; 1-Alkyl-6-vinyl-pseudo-UTP; 1-Allylpseudouridine TP; 1-Aminomethyl-pseudo-UTP; 1-Benzylpseudouridine TP; 1-Benzyloxymethylpseudouridine TP; 1-Benzyl-pseudo-UTP; 1-Biotinyl-PEG2-pseudouridine TP; 1-Biotinylpseudouridine TP; 1-Butyl-pseudo-UTP; 1-Cynamethylpseudouridine TP; 1-Cyclobutylmethyl-pseudo-UTP; 1-Cyclobutyl-pseudo-UTP; 1-Cycloheptylmethyl-pseudo-UTP; 1-Cycloheptyl-pseudo-UTP; 1-Cyclohexylmethyl-pseudo-UTP; 1-Cyclohexyl-pseudo-UTP; 1-Cyclooctylmethyl-pseudo-UTP; 1-Cyclooctylpseudo-UTP; 1-Cyclopentylmethyl-pseudo-UTP; 1-Cyclopentyl-pseudo-UTP; 1-Cyclopropylmethyl-pseudo-UTP; 1-Cyclopropyl-pseudo-UTP; 1-Ethyl-pseudo-UTP; 1-Hexyl-pseudo-UTP; 1-Homoallylpseudouridine TP; 1-Hydroxymethylpseudouridine TP; 1-isopropyl-pseudo-UTP; 1-Me-2-thio-pseudo-UTP; 1-Me-4-thio-pseudo-UTP; 1-Me-alpha-thio-pseudo-UTP; 1-Methanesulfonylmethylpseudouridine TP; 1-Methoxymethylpseudouridine TP; 1-Methyl-6-(2,2,2-Trifluoroethyl)pseudo-UTP; 1-Methyl-6-(4-morpholino)-pseudo-UTP; 1-Methyl-6-(4-thiomorpholino)-pseudo-UTP; 1-Methyl-6-(substituted phenyl)pseudo-UTP; 1-Methyl-6-amino-pseudo-UTP; 1-Methyl-6-azido-pseudo-UTP; 1-Methyl-6-bromo-pseudo-UTP; 1-Methyl-6-butyl-pseudo-UTP; 1-Methyl-6-chloro-pseudo-UTP; 1-Methyl-6-cyano-pseudo-UTP; 1-Methyl-6-dimethylamino-pseudo-UTP; 1-Methyl-6-ethoxy-pseudo-UTP; 1-Methyl-6-ethylcarboxylate-pseudo-UTP; 1-Methyl-6-ethyl-pseudo-UTP; 1-Methyl-6-fluoro-pseudo-UTP; 1-Methyl-6-formyl-pseudo-UTP; 1-Methyl-6-hydroxyamino-pseudo-UTP; 1-Methyl-6-hydroxy-pseudo-UTP; 1-Methyl-6-iodo-pseudo-UTP; 1-Methyl-6-iso-propyl-pseudo-UTP; 1-Methyl-6-methoxy-pseudo-UTP; 1-Methyl-6-methylamino-pseudo-UTP; 1-Methyl-6-phenyl-pseudo-UTP; 1-Methyl-6-propyl-pseudo-UTP; 1-Methyl-6-tert-butyl-pseudo-UTP; 1-Methyl-6-trifluoromethoxy-

pseudo-UTP; 1-Methyl-6-trifluoromethyl-pseudo-UTP; 1-Morpholinomethylpseudouridine TP; 1-Pentyl-pseudo-UTP; 1-Phenyl-pseudo-UTP; 1-Pivaloylpseudouridine TP; 1-Propargylpseudouridine TP; 1-Propyl-pseudo-UTP; 1-propynyl-pseudouridine; 1-p-tolyl-pseudo-UTP; 1-tert-Butyl-pseudo-UTP; 1-Thiomethoxymethylpseudouridine TP; 1-Thiomorpholinomethylpseudouridine TP; 1-Trifluoroacetyl pseoudouridine TP; 1-Trifluoromethyl-pseudo-UTP; 1-Vinylpseudouridine TP; 2,2'-anhydro-uridine TP; 2'-bromo-deoxyuridine TP; 2'-F-5-Methyl-2'-deoxy-UTP; 2'-OMe-5-Me-UTP; 2'-OMe-pseudo-UTP; 2'-a-Ethynyluridine TP; 2'-a-Trifluoromethyluridine TP; 2'-b-Ethynyluridine TP; 2'-b-Trifluoromethyluridine TP; 2'-Deoxy-2',2'-difluorouridine TP; 2'-Deoxy-2'-a-mercaptouridine TP; 2'-Deoxy-2'-a-thiomethoxyuridine TP; 2'-Deoxy-2'-b-aminouridine TP; 2'-Deoxy-2'-b-azidouridine TP; 2'-Deoxy-2'-b-bromouridine TP; 2'-Deoxy-2'-b-chlorouridine TP; 2'-Deoxy-2'-b-fluorouridine TP; 2'-Deoxy-2'-b-iodouridine TP; 2'-Deoxy-2'-b-mercaptouridine TP; 2'-Deoxy-2'-b-thiomethoxyuridine TP; 2-methoxy-4-thio-uridine; 2-methoxyuridine; 2'-O-Methyl-5-(1-propynyl)uridine TP; 3-Alkyl-pseudo-UTP; 4'-Azidouridine TP; 4'-Carbocyclic uridine TP; 4'-Ethynyluridine TP; 5-(1-Propynyl)ara-uridine TP; 5-(2-Furanyl)uridine TP; 5-Cyanouridine TP; 5-Dimethylaminouridine TP; 5'-Homo-uridine TP; 5-ido-2'-fluoro-deoxyuridine TP; 5-Phenylethylnyluridine TP; 5-Tri-deuteromethyl-6-deuterouridine TP; 5-Trifluoromethyl-Uridine TP; 5-Vinylauridine TP; 6-(2,2,2-Trifluoroethyl)-pseudo-UTP; 6-(4-Morpholino)-pseudo-UTP; 6-(4-Thiomorpholino)-pseudo-UTP; 6-(Substituted-Phenyl)-pseudo-UTP; 6-Amino-pseudo-UTP; 6-Azido-pseudo-UTP; 6-Bromo-pseudo-UTP; 6-Butyl-pseudo-UTP; 6-Chloro-pseudo-UTP; 6-Cyano-pseudo-UTP; 6-Dimethylamino-pseudo-UTP; 6-Ethoxy-pseudo-UTP; 6-Ethylcarboxylate-pseudo-UTP; 6-Ethyl-pseudo-UTP; 6-Fluoro-pseudo-UTP; 6-Formyl-pseudo-UTP; 6-Hydroxyamino-pseudo-UTP; 6-Hydroxy-pseudo-UTP; 6-Iodo-pseudo-UTP; 6-iso-Propyl-pseudo-UTP; 6-Methoxy-pseudo-UTP; 6-Methyl-amino-pseudo-UTP; 6-Methyl-pseudo-UTP; 6-Phenyl-pseudo-UTP; 6-Phenyl-pseudo-UTP; 6-Propyl-pseudo-UTP; 6-tert-Butyl-pseudo-UTP; 6-Trifluoromethoxy-pseudo-UTP; 6-Trifluoromethyl-pseudo-UTP; Alpha-thiopseudo-UTP; Pseudouridine 1-(4-methylbenzoic acid) TP; Pseudouridine 1-[3-(2-ethoxy)]propionic acid; Pseudouridine TP 1-[3-{2-[2-(2-ethoxy)-ethoxy]-ethoxy}]propionic acid; Pseudouridine TP 1-[3-{2-(2-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP 1-[3-{2-(2-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP 1-methylphosphonic acid; Pseudouridine TP 1-methylphosphonic acid diethyl ester; Pseudo-UTP-N1-3-propionic acid; Pseudo-UTP-N1-4-butanoic acid; Pseudo-UTP-N1-5-pentanoic acid; Pseudo-UTP-N1-6-hexanoic acid; Pseudo-UTP-N1-7-heptanoic acid; Pseudo-UTP-N1-methyl-p-benzoic acid; Pseudo-UTP-N1-p-benzoic acid; Wybutosine; Hydroxywybutosine; Isowyosine; Peroxywybutosine; undermodified hydroxywybutosine; 4-demethylwyosine; 2,6-(diamino)purine; 1-(aza)-2-(thio)-3-(aza)-phenoxyazin-1-yl; 1,3-(diaza)-2-(oxo)-phenothiazin-1-yl; 1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 1,3,5-(triaza)-2,6-(dioxa)-naphthalene; 2 (amino)purine; 2,4,5-(trimethyl)phenyl; 2' methyl, 2' amino, 2' azido, 2' fluoro-cytidine; 2' methyl, 2' amino, 2' azido, 2' fluoro-adenine; 2'methyl, 2' amino, 2' azido, 2' fluoro-uridine; 2'-amino-2'-deoxyribose; 2-amino-6-Chloro-purine; 2-aza-inosinyl; 2'-azido-2'-deoxyribose; 2'fluoro-2'-deoxyri-

bose; 2'-fluoro-modified bases; 2'-O-methyl-ribose; 2-oxo-7-aminopyridopyrimidin-3-yl; 2-oxo-pyridopyrimidine-3-yl; 2-pyridone; 3-nitropyrrrole; 3-(methyl)-7-(propynyl)isocarboxyriyl; 3-(methyl)isocarboxyriyl; 4-(fluoro)-6-(methyl)benzimidazole; 4-(methyl)benzimidazole; 4-(methyl)indolyl; 4,6-(dimethyl)indolyl; 5-nitroindole; 5-substituted pyrimidines; 5-(methyl)isocarboxyriyl; 5-nitroindole; 6-(aza)pyrimidine; 6-(azo)thymine; 6-(methyl)-7-(aza)indolyl; 6-chloro-purine; 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl; 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxyazin-1-yl; 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 7-(aza)indolyl; 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxyazin-1-yl; 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxyazin-1-yl; 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl; 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 7-(propynyl)isocarboxyriyl; 7-(propynyl)isocarboxyriyl; 7-deaza-inosinyl; 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxyazin-1-yl; 7-substituted 1,3-(diaza)-2-(oxo)-phenoxyazin-1-yl; 9-(methyl)-imidopyridinyl; Aminoindolyl; Anthracenyl; bis-ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; bis-ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; Difluorotolyl; Hypoxanthine; Imidopyridinyl; Inosinyl; Isocarboxyriyl; Isoguanisine; N2-substituted purines; N6-methyl-2-amino-purine; N6-substituted purines; N-alkylated derivative; Naphthalenyl; Nitrobenzimidazolyl; Nitroimidazolyl; Nitroindazolyl; Nitropyrazolyl; Nubularine; 06-substituted purines; O-alkylated derivative; ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; Oxoformycin TP; para-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; Pentacenyl; Phenanthracenyl; Phenyl; propynyl-7-(aza)indolyl; Pyrenyl; pyridopyrimidin-3-yl; pyridopyrimidin-3-yl; 2-oxo-7-amino-pyridopyrimidin-3-yl; pyrrolo-pyrimidin-2-on-3-yl; Pyrrolopyrimidinyl; Pyrrolopyrimidinyl; Stilbenyl; substituted 1,2,4-triazoles; Tetracycyl; Tubercidin; Xanthine; Xanthosine-5'-TP; 2-thio-zebularine; 5-aza-2-thio-zebularine; 7-deaza-2-amino-purine; pyridin-4-one ribonucleoside; 2-Amino-riboside-TP; Formycin A TP; Formycin B TP; Pyrrolidine TP; 2'-OH-ara-adenosine TP; 2'-OH-ara-cytidine TP; 2'-OH-ara-uridine TP; 2'-OH-ara-guanosine TP; 5-(2-carbomethoxyvinyl)uridine TP; and N6-(19-Amino-pentaoxonadecyl)adenosine TP.

In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) includes a combination of at least two (e.g., 2, 3, 4 or more) of the aforementioned modified nucleobases.

In some embodiments, modified nucleobases in the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) are selected from the group consisting of pseudouridine (ψ), N1-methylpseudouridine (m1 ψ), N1-ethylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihdropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine,

4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine), 5-methoxyuridine and 2'-O-methyl uridine. In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) includes a combination of at least two (e.g., 2, 3, 4 or more) of the aforementioned modified nucleobases. Each possibility represents a separate embodiment of the present invention.

In some embodiments, modified nucleobases in the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) are selected from the group consisting of 1-methyl-pseudouridine (m1 ψ), 5-methoxy-uridine (m05U), 5-methyl-cytidine (m5C), pseudouridine (ψ), α -thioguanosine and α -thio-adenosine. In some embodiments, the polynucleotide includes a combination of at least two (e.g., 2, 3, 4 or more) of the aforementioned modified nucleobases. Each possibility represents a separate embodiment of the present invention. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises pseudouridine (W) and 5-methyl-cytidine (m5C). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 1-methyl-pseudouridine (m1 ψ). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 1-methyl-pseudouridine (m1 ψ) and 5-methyl-cytidine (m5C). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 2-thiouridine (s2U). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 2-thiouridine and 5-methyl-cytidine (m5C). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 2-thiouridine (s2U). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 5-methoxy-uridine (m05U) and 5-methyl-cytidine (m5C). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 2'-O-methyl uridine. In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises 2'-O-methyl uridine and 5-methyl-cytidine (m5C). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises N6-methyl-adenosine (m6A). In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) comprises N6-methyl-adenosine (m6A) and 5-methyl-cytidine (m5C). Each possibility represents a separate embodiment of the present invention.

In some embodiments, the polynucleotide (e.g., RNA polynucleotide, such as mRNA polynucleotide) is uniformly modified (e.g., fully modified, modified throughout the entire sequence) for a particular modification. For example, a polynucleotide can be uniformly modified with 5-methyl-cytidine (m5C), meaning that all cytosine residues in the mRNA sequence are replaced with 5-methyl-cytidine (m5C). Similarly, a polynucleotide can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as any of those set forth above. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the modified nucleobase is a modified cytosine. Examples of nucleobases and nucleosides having a modified cytosine include N4-acetyl-cytidine (ac4C), 5-methyl-cytidine (m5C), 5-halo-cytidine (e.g.,

5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm5C), 1-methyl-pseudoisocytidine, 2-thio-cytidine (s2C), 2-thio-5-methyl-cytidine.

In some embodiments, a modified nucleobase is a modified uridine. Example nucleobases and nucleosides having a modified uridine include 5-cyano uridine or 4'-thio uridine. Each possibility represents a separate embodiment of the present invention.

In some embodiments, a modified nucleobase is a modified adenine. Example nucleobases and nucleosides having a modified adenine include 7-deaza-adenine, 1-methyl-adenosine (m1A), 2-methyl-adenine (m2A), N6-methyl-adenosine (m6A), and 2,6-Diaminopurine. Each possibility represents a separate embodiment of the present invention.

In some embodiments, a modified nucleobase is a modified guanine. Example nucleobases and nucleosides having a modified guanine include inosine (I), 1-methyl-inosine (m1I), wyo sine (imG), methylwyo sine (mimG), 7-deaza-guanosine, 7-cyano-7-deaza-guanosine (preQ0), 7-aminomethyl-7-deaza-guanosine (preQ1), 7-methyl-guanosine (m7G), 1-methyl-guanosine (m1G), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine. Each possibility represents a separate embodiment of the present invention.

In some embodiments, polynucleotides function as messenger RNA (mRNA). "Messenger RNA" (mRNA) refers to any polynucleotide that encodes a (at least one) polypeptide (a naturally-occurring, non-naturally-occurring, or modified polymer of amino acids) and can be translated to produce the encoded polypeptide in vitro, in vivo, in situ or ex vivo. The basic components of an mRNA molecule typically include at least one coding region, a 5' untranslated region (UTR), a 3' UTR, a 5' cap and a poly-A tail. Polynucleotides may function as mRNA but can be distinguished from wild-type mRNA in their functional and/or structural design features which serve to overcome existing problems of effective polypeptide expression using nucleic-acid based therapeutics.

The mRNA, as provided herein, comprises at least one (one or more) ribonucleic acid (RNA) polynucleotide having an open reading frame encoding at least one polypeptide of interest. In some embodiments, a RNA polynucleotide of an mRNA encodes 2-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-10, 3-9, 3-8, 3-7, 3-6, 3-5, 3-4, 4-10, 4-9, 4-8, 4-7, 4-6, 4-5, 5-10, 5-9, 5-8, 5-7, 5-6, 6-10, 6-9, 6-8, 6-7, 7-10, 7-9, 7-8, 8-10, 8-9 or 9-10 polypeptides. In some embodiments, a RNA polynucleotide of an mRNA encodes at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 polypeptides. In some embodiments, a RNA polynucleotide of an mRNA encodes at least 100 or at least 200 polypeptides.

In some embodiments, the nucleic acids are therapeutic mRNAs. As used herein, the term "therapeutic mRNA" refers to an mRNA that encodes a therapeutic protein. Therapeutic proteins mediate a variety of effects in a host cell or a subject in order to treat a disease or ameliorate the signs and symptoms of a disease. For example, a therapeutic protein can replace a protein that is deficient or abnormal, augment the function of an endogenous protein, provide a novel function to a cell (e.g., inhibit or activate an endogenous cellular activity, or act as a delivery agent for another therapeutic compound (e.g., an antibody-drug conjugate). Therapeutic mRNA may be useful for the treatment of the following diseases and conditions: bacterial infections, viral infections, parasitic infections, cell proliferation disorders, genetic disorders, and autoimmune disorders.

Thus, the structures of the invention can be used as therapeutic or prophylactic agents. They are provided for use in medicine. For example, the mRNA of the structures

described herein can be administered to a subject, wherein the polynucleotides are translated in vivo to produce a therapeutic peptide. Provided are compositions, methods, kits, and reagents for diagnosis, treatment or prevention of a disease or condition in humans and other mammals. The active therapeutic agents of the invention include the structures, cells containing structures or polypeptides translated from the polynucleotides contained in the structures.

The structures may be induced for translation in a cell, 10 tissue or organism. Such translation can be in vivo, ex vivo, in culture, or in vitro. The cell, tissue or organism is contacted with an effective amount of a composition containing a structure which contains the mRNA polynucleotides each of which has at least one translatable region 15 encoding a peptide.

An "effective amount" of the structures are provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the polynucleotide (e.g., size, and extent of modified nucleosides) and other components of the nucleic acids, and other determinants. In general, an effective amount of the nucleic acids provides an induced or boosted peptide production in the cell.

The mRNA of the present invention may be designed to 25 encode polypeptides of interest selected from any of several target categories including, but not limited to, biologics, antibodies, vaccines, therapeutic proteins or peptides, cell penetrating peptides, secreted proteins, plasma membrane proteins, cytoplasmic or cytoskeletal proteins, intracellular 30 membrane bound proteins, nuclear proteins, proteins associated with human disease, targeting moieties or those proteins encoded by the human genome for which no therapeutic indication has been identified but which nonetheless have utility in areas of research and discovery. 35 "Therapeutic protein" refers to a protein that, when administered to a cell has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

The mRNA disclosed herein, may encode one or more 40 biologics. As used herein, a "biologic" is a polypeptide-based molecule produced by the methods provided herein and which may be used to treat, cure, mitigate, prevent, or diagnose a serious or life-threatening disease or medical condition. Biologics, according to the present invention 45 include, but are not limited to, allergenic extracts (e.g. for allergy shots and tests), blood components, gene therapy products, human tissue or cellular products used in transplantation, vaccines, monoclonal antibodies, cytokines, growth factors, enzymes, thrombolytics, and immunomodulators, among others.

According to the present invention, one or more biologics currently being marketed or in development may be encoded by the mRNA of the present invention. While not wishing to be bound by theory, it is believed that incorporation of the 55 encoding polynucleotides of a known biologic into the mRNA of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and/or selectivity of the construct designs.

The mRNA disclosed herein, may encode one or more 60 antibodies or fragments thereof. The term "antibody" includes monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, multispecific antibodies (e.g., bispecific antibodies, diabodies, and single-chain molecules), as well as antibody fragments. The term "immunoglobulin" (Ig) is used interchangeably with "antibody" herein. As used herein, the term "monoclonal anti-

body" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (e.g., isomerizations, amidations) that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is(are) identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity. Chimeric antibodies of interest herein include, but are not limited to, "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey, Ape etc.) and human constant region sequences.

An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding and/or the variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2 and Fv fragments; diabodies; linear antibodies; nanobodies; single-chain antibody molecules and multispecific antibodies formed from antibody fragments.

Any of the five classes of immunoglobulins, IgA, IgD, IgE, IgG and IgM, may be encoded by the mRNA of the invention, including the heavy chains designated alpha, delta, epsilon, gamma and mu, respectively. Also included are polynucleotide sequences encoding the subclasses, gamma and mu. Hence any of the subclasses of antibodies may be encoded in part or in whole and include the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. According to the present invention, one or more antibodies or fragments currently being marketed or in development may be encoded by the mRNA of the present invention.

Antibodies encoded in the mRNA of the invention may be utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, blood, cardiovascular, CNS, poisoning (including antivenoms), dermatology, endocrinology, gastrointestinal, medical imaging, musculoskeletal, oncology, immunology, respiratory, sensory and anti-infective.

In one embodiment, mRNA disclosed herein may encode monoclonal antibodies and/or variants thereof. Variants of antibodies may also include, but are not limited to, substitutional variants, conservative amino acid substitution, insertion variants, deletional variants and/or covalent derivatives. In one embodiment, the mRNA disclosed herein may encode an immunoglobulin Fc region. In another embodiment, the mRNA may encode a variant immunoglobulin Fc region.

The mRNA disclosed herein, may encode one or more vaccine antigens. As used herein, a "vaccine antigen" is a biological preparation that improves immunity to a particular disease or infectious agent. According to the present invention, one or more vaccine antigens currently being marketed or in development may be encoded by the mRNA of the present invention. Vaccine antigens encoded in the mRNA of the invention may be utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, cancer, allergy and infectious disease.

The mRNA of the present invention may be designed to encode one or more antimicrobial peptides (AMP) or antiviral peptides (AVP). AMPs and AVPs have been isolated and described from a wide range of animals such as, but not limited to, microorganisms, invertebrates, plants, amphibians, birds, fish, and mammals. The anti-microbial polypeptides described herein may block cell fusion and/or viral entry by one or more enveloped viruses (e.g., HIV, HCV). For example, the anti-microbial polypeptide can comprise or consist of a synthetic peptide corresponding to a region, e.g., a consecutive sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids of the transmembrane subunit of a viral envelope protein, e.g., HIV-1 gp120 or gp41. The amino acid and nucleotide sequences of HIV-1 gp120 or gp41 are described in, e.g., Kuiken et al., (2008). "HIV Sequence Compendium," Los Alamos National Laboratory.

In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, 100% sequence homology to the corresponding viral protein sequence. In some embodiments, the anti-microbial polypeptide may have at least about 75% 80%, 85%, 90%, 95%, or 100% sequence homology to the corresponding viral protein sequence.

In other embodiments, the anti-microbial polypeptide may comprise or consist of a synthetic peptide corresponding to a region, e.g., a consecutive sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids of the binding domain of a capsid binding protein. In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, or 100% sequence homology to the corresponding sequence of the capsid binding protein.

The anti-microbial polypeptides described herein may block protease dimerization and inhibit cleavage of viral proproteins (e.g., HIV Gag-pol processing) into functional proteins thereby preventing release of one or more enveloped viruses (e.g., HIV, HCV). In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, 100% sequence homology to the corresponding viral protein sequence.

In other embodiments, the anti-microbial polypeptide can comprise or consist of a synthetic peptide corresponding to a region, e.g., a consecutive sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids of the binding domain of a protease binding protein. In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, 100% sequence homology to the corresponding sequence of the protease binding protein.

A non-limiting list of infectious diseases that the mRNA vaccine antigens or anti-microbial peptides may treat is presented below: human immunodeficiency virus (HIV), HIV resulting in mycobacterial infection, AIDS related Cacheux, AIDS related Cytomegalovirus infection, HIV-associated nephropathy, Lipodystrophy, AID related cryptococcal meningitis, AIDS related neutropaenia, Pneumocystis jiroveci (*Pneumocystis carinii*) infections, AID related toxoplasmosis, hepatitis A, B, C, D or E, herpes, herpes zoster (chicken pox), German measles (rubella virus), yellow fever, dengue fever etc. (flavi viruses), flu (influenza viruses), haemorrhagic infectious diseases (Marburg or Ebola viruses), bacterial infectious diseases such as Legionnaires' disease (*Legionella*), gastric ulcer (*Helicobacter*), cholera (*Vibrio*), *E. coli* infections, staphylococcal infections, *salmonella* infections or streptococcal infections, tetanus (*Clostridium tetani*), protozoan infectious diseases (ma-

laria, sleeping sickness, leishmaniasis, toxoplasmosis, i.e. infections caused by *plasmodium*, trypanosomes, *leishmania* and *toxoplasma*), diphtheria, leprosy, measles, pertussis, rabies, tetanus, tuberculosis, typhoid, varicella, diarrheal infections such as Amoebiasis, *Clostridium difficile*-associated diarrhea (CDAD), Cryptosporidiosis, Giardiasis, Cyclosporiasis and Rotaviral gastroenteritis, encephalitis such as Japanese encephalitis, Wester equine encephalitis and Tick-borne encephalitis (TBE), fungal skin diseases such as candidiasis, onychomycosis, Tinea captis/scal ring-worm, Tinea corporis/body ringworm, Tinea cruris/jock itch, sporotrichosis and Tinea pedis/Athlete's foot, Meningitis such as *Haemophilus influenza* type b (Hib), Meningitis, viral, meningococcal infections and pneumococcal infection, neglected tropical diseases such as Argentine haemorrhagic fever, Leishmaniasis, Nematode/roundworm infections, Ross river virus infection and West Nile virus (WNV) disease, Non-HIV STDs such as Trichomoniasis, Human papillomavirus (HPV) infections, sexually transmitted chlamydial diseases, Chancroid and Syphilis, Non-septic bacterial infections such as cellulitis, lyme disease, MRSA infection, *pseudomonas*, staphylococcal infections, Boutonneuse fever, Leptospirosis, Rheumatic fever, Botulism, Rickettsial disease and Mastoiditis, parasitic infections such as Cystercosis, Echinococcosis, Trematode/Fluke infections, Trichinellosis, Babesiosis, Hypodermiasis, Diphyllobothriasis and Trypanosomiasis, respiratory infections such as adenovirus infection, aspergillosis infections, avian (H5N1) influenza, influenza, RSV infections, severe acute respiratory syndrome (SARS), sinusitis, Legionellosis, Coccidioidomycosis and swine (H1N1) influenza, sepsis such as bacteraemia, sepsis/septic shock, sepsis in premature infants, urinary tract infection such as vaginal infections (bacterial), vaginal infections (fungal) and gonococcal infection, viral skin diseases such as B19 parvovirus infections, warts, genital herpes, orofacial herpes, shingles, inner ear infections, fetal cytomegalovirus syndrome, foodborn illnesses such as brucellosis (*Brucella* species), *Clostridium perfringens* (Epsilon toxin), *E. Coli* O157:H7 (*Escherichia coli*), *Salmonellosis* (*Salmonella* species), Shigellosis (Shigella), Vibriosis and Listeriosis, bioterrorism and potential epidemic diseases such as Ebola haemorrhagic fever, Lassa fever, Marburg haemorrhagic fever, plague, Anthrax Nipah virus disease, Hanta virus, Smallpox, Glanders (*Burkholderia mallei*), Melioidosis (*Burkholderia pseudomallei*), Psittacosis (*Chlamydia psittaci*), Q fever (*Coxiella burnetii*), Tularemia (*Francisella tularensis*), rubella, mumps and polio.

The mRNA disclosed herein, may encode one or more validated or "in testing" therapeutic proteins or peptides. According to the present invention, one or more therapeutic proteins or peptides currently being marketed or in development may be encoded by the mRNA of the present invention. Therapeutic proteins and peptides encoded in the mRNA of the invention may be utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, blood, cardiovascular, CNS, poisoning (including anti-venoms), dermatology, endocrinology, genetic, genitourinary, gastrointestinal, musculoskeletal, oncology, and immunology, respiratory, sensory and anti-infective.

The mRNA disclosed herein, may encode one or more cell-penetrating polypeptides. As used herein, "cell-penetrating polypeptide" or CPP refers to a polypeptide which may facilitate the cellular uptake of molecules. A cell-penetrating polypeptide of the present invention may contain one or more detectable labels. The polypeptides may be partially labeled or completely labeled throughout. The

mRNA may encode the detectable label completely, partially or not at all. The cell-penetrating peptide may also include a signal sequence. As used herein, a "signal sequence" refers to a sequence of amino acid residues bound at the amino terminus of a nascent protein during protein translation. The signal sequence may be used to signal the secretion of the cell-penetrating polypeptide.

In one embodiment, the mRNA may also encode a fusion protein. The fusion protein may be created by operably linking a charged protein to a therapeutic protein. As used herein, "operably linked" refers to the therapeutic protein and the charged protein being connected in such a way to permit the expression of the complex when introduced into the cell. As used herein, "charged protein" refers to a protein that carries a positive, negative or overall neutral electrical charge. Preferably, the therapeutic protein may be covalently linked to the charged protein in the formation of the fusion protein. The ratio of surface charge to total or surface amino acids may be approximately 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 or 0.9.

The cell-penetrating polypeptide encoded by the mRNA may form a complex after being translated. The complex may comprise a charged protein linked, e.g. covalently linked, to the cell-penetrating polypeptide.

In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but is not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the mRNA may be introduced. The cell-penetrating polypeptide may also be capable of penetrating the first cell.

In one embodiment, the mRNA may encode a cell-penetrating polypeptide which may comprise a protein-binding partner. The protein binding partner may include, but is not limited to, an antibody, a supercharged antibody or a functional fragment. The mRNA may be introduced into the cell where a cell-penetrating polypeptide comprising the protein-binding partner is introduced.

Some embodiments of the present disclosure provide a therapeutic mRNA that includes at least one ribonucleic acid (RNA) polynucleotide having an open reading frame encoding at least one antigenic polypeptide, in which the RNA polynucleotide of the RNA includes at least one chemical modification. In some embodiments, the chemical modification is selected from pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine), 5-methoxyuridine, and 2'-O-methyl uridine. Each possibility represents a separate embodiment of the present invention.

Any of the foregoing polynucleotides of the present disclosure, in some embodiments, are codon optimized. Codon optimization methods are known in the art and may be used as provided herein. Codon optimization, in some embodiments, may be used to match codon frequencies in target and host organisms to ensure proper folding; bias GC content to increase mRNA stability or reduce secondary

structures; minimize tandem repeat codons or base runs that may impair gene construction or expression; customize transcriptional and translational control regions; insert or remove protein trafficking sequences; remove/add post translation modification sites in encoded protein (e.g., glycosylation sites); add, remove or shuffle protein domains; insert or delete restriction sites; modify ribosome binding sites and mRNA degradation sites; adjust translational rates to allow the various domains of the protein to fold properly; or to reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art—non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA) and/or proprietary methods. In some embodiments, the open reading frame (ORF) sequence is optimized using optimization algorithms.

In some embodiments, a codon optimized sequence shares less than 95% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide). In some embodiments, a codon optimized sequence shares less than 90% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide. In some embodiments, a codon optimized sequence shares less than 85% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide. In some embodiments, a codon optimized sequence shares less than 80% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide. In some embodiments, a codon optimized sequence shares less than 75% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide.

In some embodiments, a codon optimized sequence shares between 65% and 85% (e.g., between about 67% and about 85% or between about 67% and about 80%) sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide. In some embodiments, a codon optimized sequence shares between 65% and 75 or about 80% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest (e.g., an antigenic protein or polypeptide.

In some embodiments a codon optimized RNA may, for instance, be one in which the levels of G/C are enhanced. The G/C-content of nucleic acid molecules may influence the stability of the RNA. RNA having an increased amount of guanine (G) and/or cytosine (C) residues may be functionally more stable than nucleic acids containing a large amount of adenine (A) and thymine (T) or uracil (U) nucleotides. WO02/098443 discloses a pharmaceutical composition containing an mRNA stabilized by sequence modifications in the translated region. Due to the degeneracy of the genetic code, the modifications work by substituting existing codons for those that promote greater RNA stability without changing the resulting amino acid. The approach is limited to coding regions of the RNA.

As used herein, when referring to polypeptides the terms “site” as it pertains to amino acid based embodiments is used synonymously with “amino acid residue” and “amino acid side chain.” As used herein when referring to polynucleotides the terms “site” as it pertains to nucleotide based embodiments is used synonymously with “nucleotide.” A site represents a position within a peptide or polypeptide or polynucleotide that may be modified, manipulated, altered, derivatized or varied within the polypeptide or polynucleotide based molecules.

As used herein the terms “termini” or “terminus” when referring to polypeptides or polynucleotides refers to an extremity of a polypeptide or polynucleotide respectively. Such extremity is not limited only to the first or final site of the polypeptide or polynucleotide but may include additional amino acids or nucleotides in the terminal regions. Polypeptide-based molecules may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH₂)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These proteins have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of 20, 30, 40, 50, or 100 amino acids which are 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% identical to any of the sequences described herein can be utilized in accordance with the disclosure. In some embodiments, a polypeptide includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein. In another example, any protein that includes a stretch of 20, 30, 40, 50, or 100 amino acids that are greater than 80%, 90%, 95%, or 100% identical to any of the sequences described herein, wherein the protein has a stretch of 5, 10, 15, 20, 25, or 30 amino acids that are less than 80%, 75%, 70%, 65% or 60% identical to any of the sequences described herein can be utilized in accordance with the disclosure.

Polypeptide or polynucleotide molecules useful in the present disclosure may share a certain degree of sequence similarity or identity with the reference molecules (e.g., reference polypeptides or reference polynucleotides), for example, with art-described molecules (e.g., engineered or designed molecules or wild-type molecules). The term “identity” as known in the art, refers to a relationship between the sequences of two or more polypeptides or polynucleotides, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between them as determined by the number of matches between strings of two or more amino acid residues or nucleic acid residues. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (e.g., “algorithms”). Identity of related peptides can be readily

calculated by known methods. "% identity" as it applies to polypeptide or polynucleotide sequences is defined as the percentage of residues (amino acid residues or nucleic acid residues) in the candidate amino acid or nucleic acid sequence that are identical with the residues in the amino acid sequence or nucleic acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent identity. Methods and computer programs for the alignment are well known in the art. It is understood that identity depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation. Generally, variants of a particular polynucleotide or polypeptide have at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, et al (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402). Another popular local alignment technique is based on the Smith-Waterman algorithm (Smith, T. F. & Waterman, M. S. (1981) "Identification of common molecular subsequences." J. Mol. Biol. 147:195-197). A general global alignment technique based on dynamic programming is the Needleman-Wunsch algorithm (Needleman, S. B. & Wunsch, C. D. (1970) "A general method applicable to the search for similarities in the amino acid sequences of two proteins." J. Mol. Biol. 48:443-453.). More recently a Fast Optimal Global Sequence Alignment Algorithm (FOGSAA) has been developed that purportedly produces global alignment of nucleotide and protein sequences faster than other optimal global alignment methods, including the Needleman-Wunsch algorithm. Other tools are described herein, specifically in the definition of "identity" below.

As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, e.g. between nucleic acid molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Polymeric molecules (e.g. nucleic acid molecules (e.g. DNA molecules and/or RNA molecules) and/or polypeptide molecules) that share a threshold level of similarity or identity determined by alignment of matching residues are termed homologous. Homology is a qualitative term that describes a relationship between molecules and can be based upon the quantitative similarity or identity. Similarity or identity is a quantitative term that defines the degree of sequence match between two compared sequences. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). Two polynucleotide sequences are considered homologous if the polypeptides they encode are at least 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4-5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4-5 uniquely specified amino acids. Two protein sequences are considered homologous if the proteins

are at least 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least 20 amino acids.

Homology implies that the compared sequences diverged in evolution from a common origin. The term "homolog" refers to a first amino acid sequence or nucleic acid sequence (e.g., gene (DNA or RNA) or protein sequence) that is related to a second amino acid sequence or nucleic acid sequence by descent from a common ancestral sequence. The term "homolog" may apply to the relationship between genes and/or proteins separated by the event of speciation or to the relationship between genes and/or proteins separated by the event of genetic duplication. "Orthologs" are genes (or proteins) in different species that evolved from a common ancestral gene (or protein) by speciation. Typically, orthologs retain the same function in the course of evolution. "Paralogs" are genes (or proteins) related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.

The term "identity" refers to the overall relatedness between polymeric molecules, for example, between polynucleotide molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleic acid sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heijne, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Grabskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleic acid sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleic acid sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those

disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., et al., *Nucleic Acids Research*, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. et al., *J. Molec. Biol.*, 215, 403 (1990).

The immunomodulatory agent may be an immunostimulatory agent or an immunoinhibitory agent. An immunostimulatory agent is an agent that stimulates an immune response (including enhancing a pre-existing immune response) in a subject to whom it is administered, whether alone or in combination with another agent. Examples include antigens, adjuvants (e.g., TLR ligands such as imiquimod, imidazoquinoline, nucleic acids comprising an unmethylated CpG dinucleotide, monophosphoryl lipid A or other lipopolysaccharide derivatives, single-stranded or double-stranded RNA, flagellin, muramyl dipeptide), cytokines including interleukins (e.g., IL-2, IL-7, IL-15 (or superagonist/mutant forms of these cytokines), IL-12, IFN-gamma, IFN-alpha, GM-CSF, FLT3-ligand, etc.), immunostimulatory antibodies (e.g., anti-CTLA-4, anti-CD28, anti-CD3, or single chain/antibody fragments of these molecules), and the like.

An immunoinhibitory agent is an agent that inhibits an immune response in a subject to whom it is administered, whether alone or in combination with another agent. Examples include steroids, retinoic acid, dexamethasone, cyclophosphamide, anti-CD3 antibody or antibody fragment, and other immunosuppressants.

Adjuvants are agents that enhance an immune response. The adjuvant may be without limitation alum (e.g., aluminum hydroxide, aluminum phosphate); saponins purified from the bark of the *Q. saponaria* tree such as QS21 (a glycolipid that elutes in the 21st peak with HPLC fractionation; Antigenics, Inc., Worcester, Mass.); poly[di(carboxy-latophenoxy) phosphazene] (PCPP polymer; Virus Research Institute, USA), Flt3 ligand, *Leishmania* elongation factor (a purified *Leishmania* protein; Corixa Corporation, Seattle, Wash.), ISCOMS (immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia), Pam3Cys, SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium), non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxy-propylene flanked by chains of polyoxyethylene, Vaxcel, Inc., Norcross, Ga.), and Montanide IMS (e.g., IMS 1312, water-based nanoparticles combined with a soluble immunostimulant, Seppic).

Adjuvants may be TLR ligands. Adjuvants that act through TLR3 include without limitation double-stranded RNA. Adjuvants that act through TLR4 include without limitation derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPLA; Ribi ImmunoChem Research, Inc., Hamilton, Mont.) and muramyl dipeptide (MDP; Ribi) and threonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland). Adjuvants that act through TLR5 include without limitation flagellin. Adjuvants that act through TLR7 and/or TLR8 include single-stranded RNA, oligoribonucleotides (ORN), synthetic low molecular weight compounds such as imidazoquinolinamines (e.g., imiquimod, resiquimod). Adjuvants acting through TLR9 include DNA of viral or bacterial origin, or synthetic oli-

godeoxynucleotides (ODN), such as CpG ODN. Another adjuvant class is phosphorothioate containing molecules such as phosphorothioate nucleotide analogs and nucleic acids containing phosphorothioate backbone linkages.

The antigen may be without limitation a cancer antigen, a self-antigen, a microbial antigen, an allergen, or an environmental antigen. The antigen may be peptide, lipid, or carbohydrate in nature, but it is not so limited.

A cancer antigen is an antigen that is expressed preferentially by cancer cells (i.e., it is expressed at higher levels in cancer cells than on non-cancer cells) and in some instances it is expressed solely by cancer cells. The cancer antigen may be expressed within a cancer cell or on the surface of the cancer cell. The cancer antigen may be MART-1/Melan-A, gp100, adenosine deaminase-binding protein (ADAbp), FAP, cyclophilin b, colorectal associated antigen (CRC)—C017-1A/GA733, carcinoembryonic antigen (CEA), CAP-1, CAP-2, etv6, AML1, prostate specific antigen (PSA), PSA-1, PSA-2, PSA-3, prostate-specific membrane antigen (PSMA), T cell receptor/CD3-zeta chain, and CD20. The cancer antigen may be selected from the group consisting of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5). The cancer antigen may be selected from the group consisting of GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9. The cancer antigen may be selected from the group consisting of BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p2iras, RCAS1, α -fetoprotein, E-cadherin, α -catenin, β -catenin, γ -catenin, p120ctn, gp100Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 ganglioside, GD2 ganglioside, human papilloma virus proteins, Smad family of tumor antigens, Imp-1, PiA, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, CD20, and c-erbB-2. Each possibility represents a separate embodiment of the present invention.

Microbial antigens are antigens derived from microbial species such as without limitation bacterial, viral, fungal, parasitic and mycobacterial species. As such, microbial antigens include bacterial antigens, viral antigens, fungal antigens, parasitic antigens, and mycobacterial antigens. Examples of bacterial, viral, fungal, parasitic and mycobacterial species are provided herein. The microbial antigen may be part of a microbial species or it may be the entire microbe.

An anti-cancer agent is an agent that at least partially inhibits the development or progression of a cancer, including inhibiting in whole or in part symptoms associated with the cancer even if only for the short term. Several anti-cancer agents can be categorized as DNA damaging agents and these include topoisomerase inhibitors (e.g., etoposide, temozolamide, topotecan, teniposide, mitoxantrone), DNA alkylating agents (e.g., cisplatin, mechlorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, busulfan, thiotepa, carbustine, lomustine, carboplatin, dacarbazine, procarbazine), DNA strand break inducing agents (e.g., bleomycin, doxorubicin, daunorubicin, idarubicin, mitomycin C), anti-microtubule agents (e.g., vincristine, vinblastine), anti-metabolic agents (e.g., cytarabine, methotrexate, hydroxyurea, 5-fluorouracil, floxuridine, 6-thioguanine,

6-mercaptopurine, fludarabine, pentostatin, chlorodeoxyadenosine), anthracyclines, vinca alkaloids, or epipodophylotoxins.

Examples of anti-cancer agents include without limitation Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Bortezomib (VELCADE); Brequinar Sodium; Bropirimine; Busulfan; Cactinomycin; Calusterone; Caracemide; Carbemter; Carboplatin (a platinum-containing regimen); Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefengol; Chlorambucil; Cirolemycin; Cisplatin (a platinum-containing regimen); Cladribine; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin; Decitabine; Dexormaplatin; Dezaguanine; Diaziquone; Docetaxel (TAXOTERE); Doxorubicin; Droxiflufen; Dromostanolone; Duazomycin; Edatrexate; Eflornithine; Elsamitrucin; Enloplatin; Enpromate; Epipropidine; Epirubicin; Erbulozole; Erlotinib (TARCEVA), Esorubicin; Estramustine; Etanidazole; Etoposide; Etoprine; Fadrozole; Fazarabine; Fenretinide; Flouxuridine; Fludarabine; 5-Fluorouracil; Fluorocitabine; Fosquidone; Fostriecin; Gefitinib (IRESSA), Gemcitabine; Hydroxyurea; Idarubicin; Ifosfamide; Ilmofosine; Imatinib mesylate (GLEEVAC); Interferon alpha-2a; Interferon alpha-2b; Interferon alpha-1; Interferon alpha-3; Interferon beta-1a; Interferon gamma-1b; Iproplatin; Irinotecan; Lanreotide; Lenalidomide (REVLIMID, REVIMID); Letrozole; Leuprolide; Liarozole; Lometrexol; Lomustine; Losoxantrone; Masoprocol; Maytansine; Mechlorethamine; Megestrol; Melengestrol; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Metoprine; Meturedopa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone; Mycophenolic Acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran; Paclitaxel; Pemetrexed (ALIMTA), Pegaspargase; Peliomycin; Pentamustine; Pentomone; Peplomycin; Perfosfamide; Pipobroman; Piposulfan; Piritrexim Isethionate; Piroxantrone; Plicamycin; Plomestane; Porfimer; Porfiromycin; Prednimustine; Procarbazine; Puromycin; Pyrazofulin; Riboprime; Rogletimide; Safingol; Semustine; Simtrazine; Sitogluside; Sparfosalate; Sparsomycin; Spirogermanium; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Tamsulosin; Taxol; Taxotere; Tecogalan; Tegafur; Teloxantrone; Temoporfin; Temozolomide (TEMODAR); Teniposide; Teroxitone; Testolactone; Thalidomide (THALOMID) and derivatives thereof, Thiamiprime; Thioguanine; Thiotepa; Tiazofurin; Tirapazamine; Topotecan; Toremifene; Trestolone; Triciridine; Trimetrexate; Triptorelin; Tubulozole; Uracil Mustard; Uredopa; Vapreotide; Verteporfin; Vinblastine; Vincristine; Vindesine; Vinepidine; Vinglycinate; Vinleurosine; Vinorelbine; Vinrosidine; Vinzolidine; Vorozole; Zeniplatin; Zinostatin; Zorubicin.

The anti-cancer agent may be an enzyme inhibitor including without limitation tyrosine kinase inhibitor, a CDK inhibitor, a MAP kinase inhibitor, or an EGFR inhibitor. The tyrosine kinase inhibitor may be without limitation Genistein (4',5,7-trihydroxyisoflavone), Tyrphostin 25 (3,4,5-trihydroxyphenyl), methylene]-propanedinitrile, Herbinycin A, Daidzein (4',7-dihydroxyisoflavone), AG-126, trans-1-(3'-carboxy-4'-hydroxyphenyl)-2-(2",5"-dihydroxyphenyl)ethane, or HDBA (2-Hydroxy-5-(2,5-Dihydroxybenzylamino)-2-hydroxybenzoic acid. The CDK inhibitor may

be without limitation p21, p27, p57, p15, p16, p18, or p19. The MAP kinase inhibitor may be without limitation KY12420 ($C_{23}H_{24}O_8$), CNI-1493, PD98059, or 4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole. The EGFR inhibitor may be without limitation erlotinib (TARCEVA), gefitinib (IRESSA), WHI-P97 (quinazoline derivative), LFM-A12 (leflunomide metabolite analog), ABX-EGF, lapatinib, canertinib, ZD-6474 (ZACTIMA), AEE788, and AG1458.

10 The anti-cancer agent may be a VEGF inhibitor including without limitation bevacizumab (AVASTIN), ranibizumab (LUCENTIS), pegaptanib (MACUGEN), sorafenib, sunitinib (SUTENT), vatalanib, ZD-6474 (ZACTIMA), anecortave (RETAANE), squalamine lactate, and semaphorin. 15 The anti-cancer agent may be an antibody or an antibody fragment including without limitation an antibody or an antibody fragment including but not limited to bevacizumab (AVASTIN), trastuzumab (HERCEPTIN), alemtuzumab (CAMPATH, indicated for B cell chronic lymphocytic leukemia), gemtuzumab (MYLOTARG, hP67.6, anti-CD33), indicated for leukemia such as acute myeloid leukemia), rituximab (RITUXAN), tositumomab (BEXXAR, anti-CD20, indicated for B cell malignancy), MDX-210 (bispecific antibody that binds simultaneously to HER-2/neu oncogene protein product and type I Fc receptors for immunoglobulin G (IgG) (Fc gamma RI)), oregovomab (OVAREX, indicated for ovarian cancer), edrecolomab (PANOUREX), daclizumab (ZENAPAX), palivizumab (SYNAGIS, indicated for respiratory conditions such as RSV infection), ibritumomab tiuxetan (ZEVALIN, indicated for Non-Hodgkin's lymphoma), cetuximab (ERBITUX), MDX-447, MDX-22, MDX-220 (anti-TAG-72), IOR-C5, IOR-T6 (anti-CD1), IOR EGF/R3, celogovab (ONCOS-CINT OV103), epratuzumab (LYMPHOCIDE), pemtumomab (THERAGYN), and Gliomab-H (indicated for brain cancer, melanoma).

A diagnostic agent, which may be referred to herein as an imaging agent, is an agent that emits signal directly or indirectly thereby allowing its detection in vivo. Diagnostic agents such as contrast agents and radioactive agents that can be detected using medical imaging techniques such as nuclear medicine scans and magnetic resonance imaging (MRI). Imaging agents for magnetic resonance imaging (MRI) include $Gd(DOTA)$, iron oxide or gold nanoparticles; imaging agents for nuclear medicine include ^{201}Tl , gamma-emitting radionuclide ^{99}mTc ; imaging agents for positron-emission tomography (PET) include positron-emitting isotopes, (^{18}F)-fluorodeoxyglucose ((^{18}FDG)), (^{18}F)-fluoride, copper-64, gadoamide, and radioisotopes of $Pb(II)$ such as 203 Pb, and 11In; imaging agents for in vivo fluorescence imaging such as fluorescent dyes or dye-conjugated nanoparticles. In other embodiments, the agent to be delivered is conjugated, or fused to, or mixed or combined with an diagnostic agent.

55 The compounds and compositions may be administered to virtually any subject type that is likely to benefit from delivery of agents as contemplated herein. Human subjects are preferred subjects in some embodiments of the invention. Subjects also include animals such as household pets (e.g., dogs, cats, rabbits, ferrets, etc.), livestock or farm animals (e.g., cows, pigs, sheep, chickens and other poultry), horses such as thoroughbred horses, laboratory animals (e.g., mice, rats, rabbits, etc.), and the like. Subjects also include fish and other aquatic species.

60 The subjects to whom the agents are delivered may be normal subjects. Alternatively they may have or may be at risk of developing a condition that can be diagnosed or that

101

can benefit from localized delivery of one or more particular agents. Such conditions include cancer (e.g., solid tumor cancers), infections (particularly infections localized to particular regions or tissues in the body), autoimmune disorders, allergies or allergic conditions, asthma, transplant rejection, and the like. In some embodiments, the subjects have been diagnosed with a genetic defect and are being administered a nucleic acid based therapeutic.

Agents may be administered systemically or locally. Agents may be administered in effective amounts. An effective amount is a dosage of the agent sufficient to provide a medically desirable result. The effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent or combination therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment.

The invention provides pharmaceutical compositions. Pharmaceutical compositions are sterile compositions that comprise agents and may comprise delivery vehicles, nanoparticles and the like, preferably in a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other subject contemplated by the invention. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the cells, nanoparticles and agent(s) are combined to facilitate administration. The components of the pharmaceutical compositions are commingled in a manner that precludes interaction that would substantially impair their desired pharmaceutical efficiency.

The compounds and compositions, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers. Pharmaceutical parenteral formulations include aqueous solutions of the ingredients. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Alternatively, suspensions of ingredients may be prepared as oil-based suspensions such as are known in the art or that will be readily apparent to those of ordinary skill in the art based on this disclosure.

This disclosure further contemplates use of LNPs together with one or more secondary agents, including agents that would normally be indicated for the subject.

In some instances, the LNPs may be administered substantially simultaneously with the secondary agents. By substantially simultaneously, it is meant that a LNP is administered to a subject close in time with the administration of the secondary agent, including for example with 1 hour, within 30 minutes, within 10 minutes, or within 5 minutes.

In some instances, the secondary agent(s) may be administered prior to the LNP. For example, the secondary agent(s) may be administered prior to and within 24 hours, or within 18 hours, or within 12 hours, or within 6 hours, or within 3 hours, or within 2 hours of the LNP administration. The secondary agent(s) may be administered 18-24 hours prior to LNP administration, or 12-18 hours prior to LNP administration, or 6-12 hours prior to LNP administration, or 2-6 hours prior to LNP administration.

102

Subjects who have been administered one or more secondary agents 2 or more hours prior to LNP administration may be referred to as having been pre-medicated with such agent(s). Subjects who have been administered one or more secondary agents within 1 hour prior to LNP administration may be referred to as having been co-medicated with such agent(s).

In some instances, the secondary agent(s) may be administered continuously to the subject, on an as needed basis or on a regular schedule (e.g., every day, every two days, etc.).

In other instances, the secondary agent may be administered before or after the administration of the LNP.

Such secondary agents may include, but are not limited to, anti-histamines, anti-platelet agents, and non-steroidal anti-inflammatory drugs. In certain embodiments, the LNPs are not formulated with and subjects are not pre- or co-medicated with a corticosteroid, such as but not limited to dexamethasone.

In certain embodiments, single secondary agents having anti-inflammatory and anti-platelet effects are used. An example of such an agent is aspirin.

In certain embodiments, a combination of aspirin, clopidrogrel (Plavix®), and an anti-histamine such as but not limited to diphenhydramine (Benadryl), fexofenadine (Allegra), loratadine (Claritin), or cetirizine is used. One or more of the secondary agents may be administered once per LNP administration while others may be administered more frequently. For example, clopidrogrel (Plavix®) may be administered once per LNP administration while aspirin and/or the anti-histamine may be administered daily.

Anti-histamines include H1 receptor antagonists and H1 receptor inverse agonists. Examples of H1 receptor antagonists include but are not limited to acrivastine, alimemazine, alimemazine tartrate, antazoline, astemizole, azatadine, azatadine maleate, azelastine, bamipine, benzquinamide, bepotastine, bepotastine besilate, bilastine bromazine, bromopheniramine, buclizine, carbinoxamine, chlorphenoxamine, chlorcyclizine, cinnopentazone histapyrrodone, chlorodiphenhydramine, chloropyramine, chlorophenamine, Chlorpromazine, cinnarizine, clemastine, clemizole, clocinizine, cyclizine, cyproheptadine, desloratadine, depropine, dexchlorpheniramine, dexbrompheniramine, dimenhydrinate, dimetindene, dimetetoride, diphenhydramine (Benadryl), piphenylpyraline, doxepin, doxylamine, ebastine, efletirizine, emdramine, emedastine, epinastine, fexofenadine (Allegra), flunarizine, homochlorcyclizine, hydroxyzine, isothioperamide, ketotifen, levocabastine (2nd generation), loratadine (Claritin), mebhydroline, meclozine, mepyramine, mequitazine, methdilazine, mirtazapine, mizolastine, niaprazine, olopatadine, orphenadrine, oxatomide, oxememazine, pemirost, phenindamine, pheniramine, phenyltoloxamine, pimethixene, piprinhydrinate, promethazine, propiomazine, pyrrobutamine, quetiapine, quifenadine, rupatadine, setastine, terfenadine, thenyldiamine, thiethylperazine, thonzylamine, tolpropamine, trimethobenzamine, tripeleannamine, triprolidine and tritoqualine.

Examples of H1 receptor inverse agonists include but are not limited to pyrilamine, cetirizine, levocetirizine, and desloratadine.

Anti-platelet agents include but are not limited to activation inhibitors, aggregation inhibitors, adhesion antagonists, anti-coagulation drugs (that do not target platelets directly), and agents that reduce platelet count or numbers.

Examples of activation inhibitors include but are not limited to (1) thrombin receptor PAR-1 inhibitors such as SCH 530348 (vorapaxar), E-5555 (atopaxar), SCH79797, FR 171113, RWJ 56110, BMS-200661, RWJ-58259,

103

SCH205831, Pipal-7 pepducin, P1pal-12 pepducin; (2) thrombin receptor PAR-4 inhibitors such as ML 354, tcY-NH₂, P4pal-10 pepducin, P4pal-i1 pepducin; (3) FSLLRY-NH₂ (PAR-2 peptide antagonist); (4) TxA2 receptor antagonists such as AH 23,848, SQ 29,548, or R 68,070, S-1452, losartan, seratrodast; (5) thromboxane receptor antagonists such as terutroban; (6) ADP P2Y12 receptor inhibitors such as ticlopidine, clopidogrel, prasugrel, ticagrelor, cangrelor, elinogrel, AZD6140, AR-C69931, CoA; (7) ADP P2Y1 receptor inhibitors such as A2P5P, A3P5P, MRS2179, MRS2279, MRS2500, palmitoyl-CoA (also acts on P2Y12), and other compounds from SAR study by Thalji et al. 2010; (8) 5-HT2A antagonists such as R-1012444, naftidrofuryl, sarpogrelate, AT-1015; (9) thromboxane synthase inhibitors such as dazoxiben, CS-518 (TXA2 synthase inhibitor), SB 203580, U63557A, imidazo (1,5-2) pyridine-5-hexanoic acid; (10) COX-1 inhibitors such as aspirin, NCX-4016, ridogrel, S18886, picotamide, ramatroban (also TXA2 receptor antagonist), SC-560, FR122047, mofezolac, P6, TFAP, ibuprofen and naproxen (also Cox-2 inhibitors); (11) COX-2 inhibitors such as trifusal (also COX-1 and PDE inhibitor), Etoricoxib, rofecoxib, celecoxib, meloxicam; and (12) PI3K inhibitors such as AZD6482.

Examples of aggregation inhibitors include but are not limited to (1) GPIa/IIa Inhibitors such as EMS16; (2) GPVI inhibitors such as monoclonal antibodies and Fab fragments of mAb 12A5; (3) GPIb/IIIa inhibitors such as abciximab, eptifibatide, tirofiban; (4) PDE inhibitors such as dipyridamole (also adenosine reuptake inhibitor), cilostazol (PDE3 inhibitor that results in increased cAMP, and activated PKA), and (5) ADP receptor antagonists. Other platelet aggregation inhibitors include aspirin, clopidogrel (Plavix®), aspirin/pravastatin, cilostazol, prasugrel, aspirin/dipyridamole, ticagrelor, cangrelor, elinogrel, dipyridamole, and ticlopidine.

Examples of adhesion antagonists (to fibrinogen) include but are not limited to C1qTNF-related protein-1, DZ-697b, RG12986.

Examples of non-platelet anti-coagulation agents include but are not limited to warfarin; Xa inhibitors such as rivaroxaban, apixaban, edoxaban, betrixaban, darexaban, otamixaban; thrombin inhibitors such as bivalirudin, hirudin, dabigatran, lepirudin, desirudin, argatroban, melagatan, dabigatran, CDSO3, FDSO3, SDSO3, and additional sulphated benzofurans allorsteric inhibitors reported by Sidhu et al. paper.

Examples of agents that reduce platelet count or number include but are not limited to (1) cAMP phosphodiesterase inhibitors (e.g., anagrelide), 6,7-dichloro-1,5-dihydroimidazo-[2,1-b]quinazolin-2(3H)-one or 6,7-dichloro-1,2,3,5-tetrahydroimidazo[2,1-b]quinazolin-2-one (U.S. Pat. Nos. 3,932,407; 4,146,718; RE31,617, Haematologica 1992 77:40-3), (2) antibodies to cell surface receptors specifically expressed by platelets or megakaryocytes such as glycoprotein IIb/IIIa receptor antibodies, (3) most chemotherapeutic anti-cancer drugs such as busulfan (Br. J. Haematol. 1986 62:229-37), hydroxyurea (N Engl J Med 1995 332:1132-6), hepsulfan, phosphorus-32 (Br J Radiol 1997 70:1169-73), pipobroman (Scand J. Haematol 1986 37:306-9), cyclophosphamide (J Cell Physiol 1982 112:222-8), certain alkylating agents and certain antimetabolites, (4) cytokines, growth factors and interleukins such as alpha-interferon (Cancer Immunol Immunother 1987 25:266-73), gamma-interferon, transforming growth factor-beta, neutrophil activating peptide-2 and its analogs (U.S. Pat. No. 5,472,944), macrophage inflammatory protein and its analogs (U.S. Pat. No. 5,306,709), (5) compounds secreted by either platelets or mega-

104

karyocytes such as platelet-factor 4 (U.S. Pat. No. 5,185,323), transforming growth factor-beta, the 12-17 kD glycoprotein produced by megakaryocytes, thrombin and thrombospondin and its amino (1-174 amino acid) terminal fragment (J Lab Clin Med 1997 129:231-8), and (6) other agents including anti-cheloid agents such as Tranilast (Rizaben) (J Dermatol 1998 25:706-9); forskolin and spleen anti-maturation factor (U.S. Pat. No. 4,088,753).

Anti-platelet agents may also be characterized as anti-thrombotic agents, fibrinolytic agents, direct thrombin inhibitors, glycoprotein IIb/IIIa receptor inhibitors, agents that bind to cellular adhesion molecules and inhibit the ability of white blood cells to attach to such molecules, calcium channel blockers, beta-adrenergic receptor blockers, cyclooxygenase-2 inhibitors, and angiotensin system inhibitors.

Anti-thrombotic agents are defined as agents which prevent the formation of a blood thrombus via a number of potential mechanisms and they include fibrinolytic agents, anti-coagulant agents, and inhibitors of platelet function.

Fibrinolytic agents are defined as agents that lyse a thrombus (e.g., a blood clot), usually through the dissolution of fibrin by enzymatic action. Examples of thrombolytic agents include but are not limited to ancrod, anistreplase, bisobrin lactate, brinolase, Hageman factor (i.e. factor XII) fragments, molsidomine, plasminogen activators such as streptokinase, tissue plasminogen activators (TPA) and urokinase, and plasmin and plasminogen. Anti-coagulant agents also include inhibitors of factor Xa, factor TFPI, factor VIIa, factor IXc, factor Va, factor VIIIa as well as inhibitors of other coagulation factors.

Anti-coagulant agents are agents which inhibit the coagulation pathway by impacting negatively upon the production, deposition, cleavage and/or activation of factors essential in the formation of a blood clot. Anti-coagulant agents include but are not limited to vitamin K antagonists such as coumarin and coumarin derivatives (e.g., warfarin sodium); glycosaminoglycans such as heparins both in unfractionated form and in low molecular weight form; ardeparin sodium, bivalirudin, bromindione, coumarin dalteparin sodium, desirudin, dicumarol, lyapolate sodium, nafamostat mesylate, phenprocoumon, sulfatide, and tinzaparin sodium.

Other “anti-coagulant” and/or “fibrinolytic” agents include Plasminogen; Streptokinase; Urokinase; Anisoylated Plasminogen-Streptokinase Activator Complex; Pro-Urokinase; (Pro-UK); rTPA (alteplase or activase; r denotes recombinant); rPro-UK; Abbokinase; Eminase; Streptase; Anagrelide Hydrochloride; Bivalirudin; Dalteparin Sodium; Danaparoid Sodium; Dazoxiben Hydrochloride; Efegatran Sulfate; Enoxaparin Sodium; Ifetroban; Ifetroban Sodium; Tinzaparin Sodium; retapase; Trisenagrel; Warfarin; Dextrans.

Still other anti-coagulant agents include, but are not limited to, Ancrod; Anticoagulant Citrate Dextrose Solution; Anticoagulant Citrate Phosphate Dextrose Adenine Solution; Anticoagulant Citrate Phosphate Dextrose Solution; Anticoagulant Heparin Solution; Anticoagulant Sodium Citrate Solution; Ardeparin Sodium; Bromindione; Desirudin; Dicumarol; Heparin Calcium; Heparin Sodium; Lyapolate Sodium; Nafamostat Mesylate; Phenprocoumon.

Clot lysing agents include, but are not limited to, tissue plasminogen activator, streptokinase, and nimodipine.

Inhibitors of platelet function are agents that impair the ability of mature platelets to perform their normal physiological roles (i.e., their normal function). Platelets are normally involved in a number of physiological processes such as adhesion, for example, to cellular and non-cellular

105

entities, aggregation, for example, for the purpose of forming a blood clot, and release of factors such as growth factors (e.g., platelet-derived growth factor (PDGF)) and platelet granular components. One subcategory of platelet function inhibitors are inhibitors of platelet aggregation which are compounds which reduce or halt the ability of platelets to associate physically with themselves or with other cellular and non-cellular components, thereby precluding the ability of a platelet to form a thrombus.

Examples of useful inhibitors of platelet function include but are not limited to acadesine, anagrelide, anipamil, argatroban, aspirin, clopidogrel, cyclooxygenase inhibitors such as nonsteroidal anti-inflammatory drugs and the synthetic compound FR-122047, danaparoid sodium, dazoxiben hydrochloride, diadenosine 5',5"-P1,P4-tetraphosphate (Ap4A) analogs, difibrotide, dilazep dihydrochloride, 1,2- and 1,3-glyceryl dinitrate, dipyridamole, dopamine and 3-methoxytyramine, efegatran sulfate, enoxaparin sodium, glucagon, glycoprotein IIb/IIIa antagonists such as Ro-43-8857 and L-700,462, ifetroban, ifetroban sodium, iloprost, isocarbacyclin methyl ester, isosorbide-5-mononitrate, itazigrel, ketanserin and BM-13.177, lamifiban, lifarizine, mol-sidomine, nifedipine, oxagrelate, PGE, platelet activating factor antagonists such as lelipafant, prostacyclin (PGI2), pyrazines, pyridinol carbamate, ReoPro (i.e., abciximab), sulfapyrazone, synthetic compounds BN-50727, BN-52021, CV-4151, E-5510, FK-409, GU-7, KB-2796, KBT-3022, KC-404, KF-4939, OP-41483, TRK-100, TA-3090, TFC-612 and ZK-36374, 2,4,5,7-tetrathiaoctane, 2,4,5,7-tetrathiaoctane 2,2-dioxide, 2,4,5-trithiahexane, theophyllin pentoxifyllin, thromboxane and thromboxane synthetase inhibitors such as picotamide and sulotroban, ticlopidine, tirofiban, trapidil and ticlopidine, trifenagrel, trilinolein, 3-substituted 5,6-bis(4-methoxyphenyl)-1,2,4-triazines, and antibodies to glycoprotein IIb/IIIa as well as those disclosed in U.S. Pat. No. 5,440,020, and anti-serotonin drugs, Clopidogrel; Sulfapyrazone; Aspirin; Dipyridamole; Clofibrate; Pyridinol Carbamate; PGE; Glucagon; Antiserotonin drugs; Caffeine; Theophyllin Pentoxifyllin; Ticlopidine.

"Direct thrombin inhibitors" include hirudin, hirugen, hirulog, agatroban, PPACK, thrombin aptamers.

"Glycoprotein IIb/IIIa receptor inhibitors" are both antibodies and non-antibodies, and include but are not limited to ReoPro (abciximab), lamifiban, tirofiban.

"Calcium channel blockers" are a chemically diverse class of compounds having important therapeutic value in the control of a variety of diseases (Fleckenstein, *Cir. Res.* v. 52, (suppl. 1), p. 13-16 (1983); Fleckenstein, *Experimental Facts and Therapeutic Prospects*, John Wiley, New York (1983); McCall, D., *Curr Pract Cardiol*, v. 10, p. 1-11 (1985)). Calcium channel blockers are a heterogeneous group of drugs that prevent or slow the entry of calcium into cells by regulating cellular calcium channels. (Remington, *The Science and Practice of Pharmacy*, Nineteenth Edition, Mack Publishing Company, Eaton, PA, p. 963 (1995)). Most of the currently available calcium channel blockers, and useful according to the present invention, belong to one of three major chemical groups of drugs, the dihydropyridines, such as nifedipine, the phenyl alkyl amines, such as verapamil, and the benzothiazepines, such as diltiazem. Other calcium channel blockers useful according to the invention, include, but are not limited to, amrinone, amlodipine, bendcyclane, felodipine, fendiline, flunarizine, isradipine, nicardipine, nimodipine, perhexilene, gallopamil, tiapamil and tiapamil analogues (such as 1993RO-11-2933), phentytoin,

106

barbiturates, and the peptides dynorphin, omega-conotoxin, and omega-agatoxin, and the like and/or pharmaceutically acceptable salts thereof.

"Beta-adrenergic receptor blocking agents" are a class of drugs that antagonize the cardiovascular effects of catecholamines in angina pectoris, hypertension, and cardiac arrhythmias. Beta-adrenergic receptor blockers include, but are not limited to, atenolol, acebutolol, alprenolol, befunolol, betaxolol, bunitrolol, carteolol, celiprolol, hydroxalol, indenolol, labetalol, levobunolol, mepindolol, methypranol, metindol, metoprolol, metrizoranolol, oxprenolol, pindolol, propranolol, practolol, practolol, sotalolnadolol, tiprenolol, tomalolol, timolol, bupranolol, penbutolol, trimepranol, 2-(3-(1,1-dimethylethyl)-amino-2-hydroxypropoxy)-3-pyridenecarbonitrilHCl, 1-butylamino-3-(2,5-dichlorophenoxy)-2-propanol, 1-isopropylamino-3-(4-(2-cyclopropylmethoxyethyl)phenoxy)-2-propanol, 3-isopropylamino-1-(7-methylindan-4-yloxy)-2-butanol, 2-(3-t-butylamino-2-hydroxy-propylthio)-4-(5-carbamoyl-2-thienyl)thiazol, 7-(2-hydroxy-3-t-butylaminoproxy)phthalide. The above-identified compounds can be used as isomeric mixtures, or in their respective levorotating or dextrorotating form.

Cyclooxygenase-2 (COX-2) is a recently identified form of a cyclooxygenase. "Cyclooxygenase" is an enzyme complex present in most tissues that produces various prostaglandins and thromboxanes from arachidonic acid. Non-steroidal, anti-inflammatory drugs exert most of their anti-inflammatory, analgesic and antipyretic activity and inhibit hormone-induced uterine contractions and certain types of cancer growth through inhibition of the cyclooxygenase (also known as prostaglandin G/H synthase and/or prostaglandin-endoperoxide synthase). Initially, only one form of cyclooxygenase was known, the "constitutive enzyme" or cyclooxygenase-1 (COX-1). It and was originally identified in bovine seminal vesicles.

Cyclooxygenase-2 (COX-2) has been cloned, sequenced and characterized initially from chicken, murine and human sources (See, e.g., U.S. Pat. No. 5,543,297, issued Aug. 6, 1996 to Cromlish, et al., and assigned to Merck Frosst Canada, Inc., Kirkland, CA, entitled: "Human cyclooxygenase-2 cDNA and assays for evaluating cyclooxygenase-2 activity").

A number of selective "COX-2 inhibitors" are known in the art. These include, but are not limited to, COX-2 inhibitors described in U.S. Pat. No. 5,474,995 "Phenyl heterocycles as cox-2 inhibitors"; U.S. Pat. No. 5,521,213 "Diaryl bicyclic heterocycles as inhibitors of cyclooxygenase-2"; U.S. Pat. No. 5,536,752 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,550,142 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,552,422 "Aryl substituted 5,5 fused aromatic nitrogen compounds as anti-inflammatory agents"; U.S. Pat. No. 5,604,253 "N-benzylindol-3-yl propanoic acid derivatives as cyclooxygenase inhibitors"; U.S. Pat. No. 5,604,260 "5-methanesulfonamido-1-indanones as an inhibitor of cyclooxygenase-2"; U.S. Pat. No. 5,639,780 N-benzyl indol-3-yl butanoic acid derivatives as cyclooxygenase inhibitors"; U.S. Pat. No. 5,677,318 Diphenyl-1,2,3-thiadiazoles as anti-inflammatory agents"; U.S. Pat. No. 5,691,374 "Diaryl-5-oxygenated-2-(5H)-furanes as COX-2 inhibitors"; U.S. Pat. No. 5,698,584 "3,4-diaryl-2-hydroxy-2,5-dihydrofurans as prodrugs to COX-2 inhibitors"; U.S. Pat. No. 5,710,140 "Phenyl heterocycles as COX-2 inhibitors"; U.S. Pat. No. 5,733,909 "Diphenyl stilbenes as prodrugs to COX-2 inhibitors"; U.S. Pat. No. 5,789,413 "Alkylated styrenes as prodrugs to COX-2 inhibitors"; U.S. Pat. No. 5,817,700 "Bisaryl

107

cyclobutenes derivatives as cyclooxygenase inhibitors”; U.S. Pat. No. 5,849,943 “Stilbene derivatives useful as cyclooxygenase-2 inhibitors”; U.S. Pat. No. 5,861,419 “Substituted pyridines as selective cyclooxygenase-2 inhibitors”; U.S. Pat. No. 5,922,742 “Pyridinyl-2-cyclopenten-1-ones as selective cyclooxygenase-2 inhibitors”; U.S. Pat. No. 5,925,631 “Alkylated styrenes as prodrugs to COX-2 inhibitors”; all of which are commonly assigned to Merck Frosst Canada, Inc. (Kirkland, CA). Additional COX-2 inhibitors are also described in U.S. Pat. No. 5,643,933, assigned to G. D. Searle & Co. (Skokie, IL), entitled: “Substituted sulfonylphenylheterocycles as cyclooxygenase-2 and 5-lipoxygenase inhibitors.” Aspirin is an example of a COX-2 inhibitor.

A number of the above-identified COX-2 inhibitors are prodrugs of selective COX-2 inhibitors, and exert their action by conversion in vivo to the active and selective COX-2 inhibitors. The active and selective COX-2 inhibitors formed from the above-identified COX-2 inhibitor prodrugs are described in detail in WO 95/00501, published Jan. 5, 1995, WO 95/18799, published Jul. 13, 1995 and U.S. Pat. No. 5,474,995, issued Dec. 12, 1995. Given the teachings of U.S. Pat. No. 5,543,297, entitled: “Human cyclooxygenase-2 cDNA and assays for evaluating cyclooxygenase-2 activity,” a person of ordinary skill in the art would be able to determine whether an agent is a selective COX-2 inhibitor or a precursor of a COX-2 inhibitor, and therefore part of the present invention.

Non-steroidal anti-inflammatory drugs include but are not limited to naproxen sodium, diclofenac, sulindac, oxaprozin, diflunisal, aspirin, piroxicam, indomethacin, etodolac, ibuprofen, fenoprofen, ketoprofen, mefenamic acid, nabumetone, tolmetin sodium, and ketorolac tromethamine.

In certain embodiments, the LNP may comprise one or more secondary agents that inhibit immune responses induced by LNPs, e.g., inhibit the binding to or activity of sensors e.g., natural IgM production, natural IgG production, activation of B1a cells, activation of B1b cells, and/or activation of platelets and/or dendritic cells or the activity or production of any effectors. The secondary agents are referred to alternatively as agents that inhibit immune responses induced by LNPs. In some instances, the secondary agent may inhibit the production of natural IgM that binds the LNPs, or neutralize such natural IgMs. In other instances, the secondary agent may inhibit activation of B1a cells or remove B1a cells. For example, such a secondary agent may inhibit a surface receptor of B1a cells, including, but not limited to CD36. Alternatively or in addition, the secondary agent may interfere with the binding of IgM to its target. In other embodiments, the secondary agent may inhibit the production of natural IgG that binds the LNPs, or neutralize such natural IgGs or may interfere with the binding of IgG to its target. In other instances, the secondary agent may inhibit activation of B1b cells or remove B1b cells.

In some embodiments, the secondary agent may be an agent that inhibits the production of natural IgM, IgG, and/or activation of B1a and/or B1b cells by LNPs. Such agents may be antagonists of a surface receptor of B1a cells (e.g., CD36 and C_{5a}) or B1b cells, for examples, antibodies or small molecule inhibitors that bind the surface receptor and interfere with its binding to its cognate ligands (e.g., lipid component such as phosphatidylcholine in certain LNPs).

In other embodiments, the secondary agent may be an agent that inhibits the activation of platelets and/or complement system (classical pathway or alternative pathway) by LNPs. Such agents may be CD36 antagonists, TLR antago-

108

nists, or antagonists of any component in the complement cascade. Such antagonists may be antagonistic antibodies specific to one of the targets. In some examples, the antagonists may be a protease inhibitor that targets one or more of the serine protease component in the complement system. Other CD36 antagonists include, but are not limited to, salvianolic acid or metabolites thereof (e.g., RA and DSS), 3-cinnamoyl indole, 13-pentyl berberine, hexarelin, or certain fatty acids such as DHA.

It is to be understood that the disclosure contemplates use of one or more of the foregoing secondary agents with any of the LNP provided herein.

Chemical Definitions

Definitions of specific functional groups and chemical terms are described in more detail below. The chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, *Handbook of Chemistry and Physics*, 75th Ed., inside cover, and specific functional groups are generally defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in *Organic Chemistry*, Thomas Sorrell, University Science Books, Sausalito, 1999; Smith and March, *March's Advanced Organic Chemistry*, 5th Edition, John Wiley & Sons, Inc., New York, 2001; Larock, *Comprehensive Organic Transformations*, VCH Publishers, Inc., New York, 1989; and Carruthers, *Some Modern Methods of Organic Synthesis*, 3rd Edition, Cambridge University Press, Cambridge, 1987.

Compounds described herein can comprise one or more asymmetric centers, and thus can exist in various stereoisomeric forms, e.g., enantiomers and/or diastereomers. For example, the compounds described herein can be in the form of an individual enantiomer, diastereomer or geometric isomer, or can be in the form of a mixture of stereoisomers, including racemic mixtures and mixtures enriched in one or more stereoisomer. Isomers can be isolated from mixtures by methods known to those skilled in the art, including chiral high pressure liquid chromatography (HPLC) and the formation and crystallization of chiral salts; or preferred isomers can be prepared by asymmetric syntheses. See, for example, Jacques et al., *Enantiomers, Racemates and Resolutions* (Wiley Interscience, New York, 1981); Wilen et al., *Tetrahedron* 33:2725 (1977); Eliel, E. L. *Stereochemistry of Carbon Compounds* (McGraw-Hill, N Y, 1962); and Wilen, S. H., *Tables of Resolving Agents and Optical Resolutions* p. 268 (E. L. Eliel, Ed., Univ. of Notre Dame Press, Notre Dame, IN 1972). The invention additionally encompasses compounds as individual isomers substantially free of other isomers, and alternatively, as mixtures of various isomers.

In a formula, ~ is a single bond where the stereochemistry of the moieties immediately attached thereto is not specified, --- is absent or a single bond, and == or == is a single or double bond.

Unless otherwise stated, structures depicted herein are also meant to include compounds that differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of hydrogen by deuterium or tritium, replacement of ¹⁹F with ¹⁸F, or the replacement of ¹²C with ¹³C or ¹⁴C are within the scope of the disclosure. Such compounds are useful, for example, as analytical tools or probes in biological assays.

When a range of values is listed, it is intended to encompass each value and sub-range within the range. For example

109

“C₁₋₆ alkyl” is intended to encompass, C₁, C₂, C₃, C₄, C₅, C₆, C₁₋₆, C₁₋₅, C₁₋₄, C₁₋₃, C₁₋₂, C₂₋₆, C₂₋₅, C₂₋₄, C₂₋₃, C₃₋₆, C₃₋₅, C₃₋₄, C₄₋₆, C₄₋₅, and C₅₋₆ alkyl.

The term “aliphatic” refers to alkyl, alkenyl, alkynyl, and carbocyclic groups. Likewise, the term “heteroaliphatic” refers to heteroalkyl, heteroalkenyl, heteroalkynyl, and heterocyclic groups.

The term “alkyl” refers to a radical of a straight-chain or branched saturated hydrocarbon group having from 1 to 10 carbon atoms (“C₁₋₁₀ alkyl”). In some embodiments, an alkyl group has 1 to 9 carbon atoms (“C₁₋₉ alkyl”). In some embodiments, an alkyl group has 1 to 8 carbon atoms (“C₁₋₈ alkyl”). In some embodiments, an alkyl group has 1 to 7 carbon atoms (“C₁₋₇ alkyl”). In some embodiments, an alkyl group has 1 to 6 carbon atoms (“C₁₋₆ alkyl”). In some embodiments, an alkyl group has 1 to 5 carbon atoms (“C₁₋₅ alkyl”). In some embodiments, an alkyl group has 1 to 4 carbon atoms (“C₁₋₄ alkyl”). In some embodiments, an alkyl group has 1 to 3 carbon atoms (“C₁₋₃ alkyl”). In some embodiments, an alkyl group has 1 to 2 carbon atoms (“C₁₋₂ alkyl”). In some embodiments, an alkyl group has 1 carbon atom (“C₁ alkyl”). In some embodiments, an alkyl group has 2 to 6 carbon atoms (“C₂₋₆ alkyl”). Examples of C₁₋₆ alkyl groups include methyl (C₁), ethyl (C₂), propyl (C₃) (e.g., n-propyl, isopropyl), butyl (C₄) (e.g., n-butyl, tert-butyl, sec-butyl, iso-butyl), pentyl (C₅) (e.g., n-pentyl, 3-pentanyl, amyl, neopentyl, 3-methyl-2-butanyl, tertiary amyl), and hexyl (C₆) (e.g., n-hexyl). Additional examples of alkyl groups include n-heptyl (C₇), n-octyl (C₈), and the like. Unless otherwise specified, each instance of an alkyl group is independently unsubstituted (an “unsubstituted alkyl”) or substituted (a “substituted alkyl”) with one or more substituents (e.g., halogen, such as F). In certain embodiments, the alkyl group is an unsubstituted C₁₋₁₀ alkyl (such as unsubstituted C₁₋₆ alkyl, e.g., —CH₃ (Me), unsubstituted ethyl (Et), unsubstituted propyl (Pr, e.g., unsubstituted n-propyl (n-Pr), unsubstituted isopropyl (i-Pr)), unsubstituted butyl (Bu, e.g., unsubstituted n-butyl (n-Bu), unsubstituted tert-butyl (tert-Bu or t-Bu), unsubstituted sec-butyl (sec-Bu), unsubstituted isobutyl (i-Bu)). In certain embodiments, the alkyl group is a substituted C₁₋₁₀ alkyl (such as substituted C₁₋₆ alkyl, e.g., —CF₃, Bn).

The term “haloalkyl” is a substituted alkyl group, wherein one or more of the hydrogen atoms are independently replaced by a halogen, e.g., fluoro, bromo, chloro, or iodo. In some embodiments, the haloalkyl moiety has 1 to 8 carbon atoms (“C₁₋₈ haloalkyl”). In some embodiments, the haloalkyl moiety has 1 to 6 carbon atoms (“C₁₋₆ haloalkyl”). In some embodiments, the haloalkyl moiety has 1 to 4 carbon atoms (“C₁₋₄ haloalkyl”). In some embodiments, the haloalkyl moiety has 1 to 3 carbon atoms (“C₁₋₃ haloalkyl”). In some embodiments, the haloalkyl moiety has 1 to 2 carbon atoms (“C₁₋₂ haloalkyl”). Examples of haloalkyl groups include —CHF₂, —CH₂F, —CF₃, —CH₂CF₃, —CF₂CF₃, —CF₂CF₂CF₃, —CCl₃, —CFCI₂, —CF₂Cl, and the like.

The term “heteroalkyl” refers to an alkyl group, which further includes at least one heteroatom (e.g., 1, 2, 3, or 4 heteroatoms) selected from oxygen, nitrogen, or sulfur within (i.e., inserted between adjacent carbon atoms of) and/or placed at one or more terminal position(s) of the parent chain. In certain embodiments, a heteroalkyl group refers to a saturated group having from 1 to 10 carbon atoms and 1 or more heteroatoms within the parent chain (“heteroC₁₋₁₀ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 9 carbon atoms and 1 or more heteroatoms within the parent chain (“heteroC₁₋₉

110

alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 8 carbon atoms and 1 or more heteroatoms within the parent chain (“heteroC₁₋₈ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 7 carbon atoms and 1 or more heteroatoms within the parent chain (“heteroC₁₋₇ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 6 carbon atoms and 1 or more heteroatoms within the parent chain (“heteroC₁₋₆ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 5 carbon atoms and 1 or 2 heteroatoms within the parent chain (“heteroC₁₋₅ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 4 carbon atoms and 1 or 2 heteroatoms within the parent chain (“heteroC₁₋₄ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 to 3 carbon atoms and 1 heteroatom within the parent chain (“heteroC₁₋₃ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 1 carbon atom and 1 heteroatom (“heteroC₁ alkyl”). In some embodiments, a heteroalkyl group is a saturated group having 2 to 6 carbon atoms and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₆ alkyl”). Unless otherwise specified, each instance of a heteroalkyl group is independently unsubstituted (an “unsubstituted heteroalkyl”) or substituted (a “substituted heteroalkyl”) with one or more substituents. In certain embodiments, the heteroalkyl group is an unsubstituted heteroC₁₋₁₀ alkyl. In certain embodiments, the heteroalkyl group is a substituted heteroC₁₋₁₀ alkyl.

The term “alkenyl” refers to a radical of a straight-chain or branched hydrocarbon group having from 2 to 10 carbon atoms and one or more carbon-carbon double bonds (e.g., 1, 2, 3, or 4 double bonds). In some embodiments, an alkenyl group has 2 to 9 carbon atoms (“C₂₋₉ alkenyl”). In some embodiments, an alkenyl group has 2 to 8 carbon atoms (“C₂₋₈ alkenyl”). In some embodiments, an alkenyl group has 2 to 7 carbon atoms (“C₂₋₇ alkenyl”). In some embodiments, an alkenyl group has 2 to 6 carbon atoms (“C₂₋₆ alkenyl”). In some embodiments, an alkenyl group has 2 to 5 carbon atoms (“C₂₋₅ alkenyl”). In some embodiments, an alkenyl group has 2 to 4 carbon atoms (“C₂₋₄ alkenyl”). In some embodiments, an alkenyl group has 2 to 3 carbon atoms (“C₂₋₃ alkenyl”). In some embodiments, an alkenyl group has 2 carbon atoms (“C₂ alkenyl”). The one or more carbon-carbon double bonds can be internal (such as in 2-butene) or terminal (such as in 1-butene). Examples of C₂₋₄ alkenyl groups include ethenyl (C₂), 1-propenyl (C₃), 2-propenyl (C₃), 1-butene (C₄), 2-butene (C₄), butadienyl (C₄), and the like. Examples of C₂₋₆ alkenyl groups include the aforementioned C₂₋₄ alkenyl groups as well as pentenyl (C₅), pentadienyl (C₅), hexenyl (C₆), and the like. Additional examples of alkenyl include heptenyl (C₇), octenyl (C₈), octatrienyl (C₈), and the like. Unless otherwise specified, each instance of an alkenyl group is independently unsubstituted (an “unsubstituted alkenyl”) or substituted (a “substituted alkenyl”) with one or more substituents. In certain embodiments, the alkenyl group is an unsubstituted C₂₋₁₀ alkenyl. In certain embodiments, the alkenyl group is a substituted C₂₋₁₀ alkenyl. In an alkenyl group, a C=C double bond for which the stereochemistry is not specified (e.g., —CH=CHCH₃ or



may be an (E)- or (Z)-double bond.

111

The term “heteroalkenyl” refers to an alkenyl group, which further includes at least one heteroatom (e.g., 1, 2, 3, or 4 heteroatoms) selected from oxygen, nitrogen, or sulfur within (i.e., inserted between adjacent carbon atoms of) and/or placed at one or more terminal position(s) of the parent chain. In certain embodiments, a heteroalkenyl group refers to a group having from 2 to 10 carbon atoms, at least one double bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₁₀ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 9 carbon atoms at least one double bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₉ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 8 carbon atoms, at least one double bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₈ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 7 carbon atoms, at least one double bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₇ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 6 carbon atoms, at least one double bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₆ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 5 carbon atoms, at least one double bond, and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₅ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 4 carbon atoms, at least one double bond, and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₄ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 3 carbon atoms, at least one double bond, and 1 heteroatom within the parent chain (“heteroC₂₋₃ alkenyl”). In some embodiments, a heteroalkenyl group has 2 to 6 carbon atoms, at least one double bond, and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₆ alkenyl”). Unless otherwise specified, each instance of a heteroalkenyl group is independently unsubstituted (an “unsubstituted heteroalkenyl”) or substituted (a “substituted heteroalkenyl”) with one or more substituents. In certain embodiments, the heteroalkenyl group is an unsubstituted heteroC₂₋₁₀ alkenyl. In certain embodiments, the heteroalkenyl group is a substituted heteroC₂₋₁₀ alkenyl.

The term “alkynyl” refers to a radical of a straight-chain or branched hydrocarbon group having from 2 to 10 carbon atoms and one or more carbon-carbon triple bonds (e.g., 1, 2, 3, or 4 triple bonds) (“C₂₋₁₀ alkynyl”). In some embodiments, an alkynyl group has 2 to 9 carbon atoms (“C₂₋₉ alkynyl”). In some embodiments, an alkynyl group has 2 to 8 carbon atoms (“C₂₋₈ alkynyl”). In some embodiments, an alkynyl group has 2 to 7 carbon atoms (“C₂₋₇ alkynyl”). In some embodiments, an alkynyl group has 2 to 6 carbon atoms (“C₂₋₆ alkynyl”). In some embodiments, an alkynyl group has 2 to 5 carbon atoms (“C₂₋₅ alkynyl”). In some embodiments, an alkynyl group has 2 to 4 carbon atoms (“C₂₋₄ alkynyl”). In some embodiments, an alkynyl group has 2 to 3 carbon atoms (“C₂₋₃ alkynyl”). In some embodiments, an alkynyl group has 2 carbon atoms (“C₂ alkynyl”). The one or more carbon-carbon triple bonds can be internal (such as in 2-butynyl) or terminal (such as in 1-butynyl). Examples of C₂₋₄ alkynyl groups include, without limitation, ethynyl (C₂), 1-propynyl (C₃), 2-propynyl (C₃), 1-butynyl (C₄), 2-butynyl (C₄), and the like. Examples of C₂₋₆ alkynyl groups include the aforementioned C₂₋₄ alkynyl groups as well as pentynyl (C₅), hexynyl (C₆), and the like. Additional examples of alkynyl include heptynyl (C₇), octynyl (C₈), and the like. Unless otherwise specified, each instance of an alkynyl group is independently unsubstituted (an “unsubstituted alkynyl”) or substituted (a “substituted alkynyl”) with one or more substituents. In certain embodiments, the alky-

112

nyl group is an unsubstituted C₂₋₁₀ alkynyl. In certain embodiments, the alkynyl group is a substituted C₂₋₁₀ alkynyl.

The term “heteroalkynyl” refers to an alkynyl group, which further includes at least one heteroatom (e.g., 1, 2, 3, or 4 heteroatoms) selected from oxygen, nitrogen, or sulfur within (i.e., inserted between adjacent carbon atoms of) and/or placed at one or more terminal position(s) of the parent chain. In certain embodiments, a heteroalkynyl group refers to a group having from 2 to 10 carbon atoms, at least one triple bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₁₀ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 9 carbon atoms, at least one triple bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₉ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 8 carbon atoms, at least one triple bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₈ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 7 carbon atoms, at least one triple bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₇ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 6 carbon atoms, at least one triple bond, and 1 or more heteroatoms within the parent chain (“heteroC₂₋₆ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 5 carbon atoms, at least one triple bond, and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₅ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 4 carbon atoms, at least one triple bond, and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₄ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 3 carbon atoms, at least one triple bond, and 1 heteroatom within the parent chain (“heteroC₂₋₃ alkynyl”). In some embodiments, a heteroalkynyl group has 2 to 6 carbon atoms, at least one triple bond, and 1 or 2 heteroatoms within the parent chain (“heteroC₂₋₆ alkynyl”). Unless otherwise specified, each instance of a heteroalkynyl group is independently unsubstituted (an “unsubstituted heteroalkynyl”) or substituted (a “substituted heteroalkynyl”) with one or more substituents. In certain embodiments, the heteroalkynyl group is an unsubstituted heteroC₂₋₁₀ alkynyl. In certain embodiments, the heteroalkynyl group is a substituted heteroC₂₋₁₀ alkynyl.

The term “carbocyclyl” or “carbocyclic” refers to a radical of a non-aromatic cyclic hydrocarbon group having from 3 to 14 ring carbon atoms (“C₃₋₁₄ carbocyclyl”) and zero heteroatoms in the non-aromatic ring system. In some embodiments, a carbocyclyl group has 3 to 10 ring carbon atoms (“C₃₋₁₀ carbocyclyl”). In some embodiments, a carbocyclyl group has 3 to 8 ring carbon atoms (“C₃₋₈ carbocyclyl”). In some embodiments, a carbocyclyl group has 3 to 7 ring carbon atoms (“C₃₋₇ carbocyclyl”). In some embodiments, a carbocyclyl group has 3 to 6 ring carbon atoms (“C₃₋₆ carbocyclyl”). In some embodiments, a carbocyclyl group has 4 to 6 ring carbon atoms (“C₄₋₆ carbocyclyl”). In some embodiments, a carbocyclyl group has 5 to 6 ring carbon atoms (“C₅₋₆ carbocyclyl”). In some embodiments, a carbocyclyl group has 5 to 10 ring carbon atoms (“C₅₋₁₀ carbocyclyl”). Exemplary C₃₋₆ carbocyclyl groups include, without limitation, cyclopropyl (C₃), cyclopropenyl (C₃), cyclobutyl (C₄), cyclobutenyl (C₄), cyclopentyl (C₅), cyclopentenyl (C₅), cyclohexyl (C₆), cyclohexenyl (C₆), cyclohexadienyl (C₆), and the like. Exemplary C₃₋₈ carbocyclyl groups include, without limitation, the aforementioned C₃₋₆ carbocyclyl groups as well as cycloheptyl (C₇), cycloheptenyl (C₇), cycloheptadienyl (C₇), cycloheptatrienyl (C₇), cyclooctyl (C₈), cyclooctenyl (C₈), bicyclo[2.2.1]heptanyl (C₇), bicyclo[2.2.2]octanyl (C₈), and the like. Exemplary C₃₋₁₀ carbocyclyl groups include, without limitation, the

aforementioned C₃₋₈ carbocyclyl groups as well as cyclo-nonyl (C₉), cyclononenyl (C₉), cyclodecyl (C₁₀), cyclodec-enyl (C₁₀), octahydro-1H-indenyl (C₉), decahydronaphthalenyl (C₁₀), spiro[4.5]decanyl (C₁₀), and the like. As the foregoing examples illustrate, in certain embodiments, the carbocyclyl group is either monocyclic ("monocyclic carbocyclyl") or polycyclic (e.g., containing a fused, bridged or spiro ring system such as a bicyclic system ("bicyclic carbocyclyl") or tricyclic system ("tricyclic carbocyclyl")) and can be saturated or can contain one or more carbon-carbon double or triple bonds. "Carbocyclyl" also includes ring systems wherein the carbocyclyl ring, as defined above, is fused with one or more aryl or heteroaryl groups wherein the point of attachment is on the carbocyclyl ring, and in such instances, the number of carbons continue to designate the number of carbons in the carbocyclic ring system. Unless otherwise specified, each instance of a carbocyclyl group is independently unsubstituted (an "unsubstituted carbocyclyl") or substituted (a "substituted carbocyclyl") with one or more substituents. In certain embodiments, the carbocyclyl group is an unsubstituted C₃₋₁₄ carbocyclyl. In certain embodiments, the carbocyclyl group is a substituted C₃₋₁₄ carbocyclyl.

In some embodiments, "carbocyclyl" is a monocyclic, saturated carbocyclyl group having from 3 to 14 ring carbon atoms ("C₃₋₁₄ cycloalkyl"). In some embodiments, a cycloalkyl group has 3 to 10 ring carbon atoms ("C₃₋₁₀ cycloalkyl"). In some embodiments, a cycloalkyl group has 3 to 8 ring carbon atoms ("C₃₋₈ cycloalkyl"). In some embodiments, a cycloalkyl group has 3 to 6 ring carbon atoms ("C₃₋₆ cycloalkyl"). In some embodiments, a cycloalkyl group has 4 to 6 ring carbon atoms ("C₄₋₆ cycloalkyl"). In some embodiments, a cycloalkyl group has 5 to 6 ring carbon atoms ("C₅₋₆ cycloalkyl"). In some embodiments, a cycloalkyl group has 5 to 10 ring carbon atoms ("C₅₋₁₀ cycloalkyl"). Examples of C₅₋₆ cycloalkyl groups include cyclopentyl (C₅) and cyclohexyl (C₆). Examples of C₃₋₆ cycloalkyl groups include the aforementioned C₃₋₆ cycloalkyl groups as well as cyclopropyl (C₃) and cyclobutyl (C₄). Examples of C₃₋₈ cycloalkyl groups include the aforementioned C₃₋₆ cycloalkyl groups as well as cycloheptyl (C₇) and cyclooctyl (C₈). Unless otherwise specified, each instance of a cycloalkyl group is independently unsubstituted (an "unsubstituted cycloalkyl") or substituted (a "substituted cycloalkyl") with one or more substituents. In certain embodiments, the cycloalkyl group is an unsubstituted C₃₋₁₄ cycloalkyl. In certain embodiments, the cycloalkyl group is a substituted C₃₋₁₄ cycloalkyl.

The term "heterocyclyl" or "heterocyclic" refers to a radical of a 3- to 14-membered non-aromatic ring system having ring carbon atoms and 1 to 4 ring heteroatoms, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur ("3-14 membered heterocyclyl"). In heterocyclyl groups that contain one or more nitrogen atoms, the point of attachment can be a carbon or nitrogen atom, as valency permits. A heterocyclyl group can either be monocyclic ("monocyclic heterocyclyl") or polycyclic (e.g., a fused, bridged or spiro ring system such as a bicyclic system ("bicyclic heterocyclyl") or tricyclic system ("tricyclic heterocyclyl")), and can be saturated or can contain one or more carbon-carbon double or triple bonds. Heterocyclyl polycyclic ring systems can include one or more heteroatoms in one or both rings. "Heterocyclyl" also includes ring systems wherein the heterocyclyl ring, as defined above, is fused with one or more carbocyclyl groups wherein the point of attachment is either on the carbocyclyl or heterocyclyl ring, or ring systems wherein the heterocycl-

cyl ring, as defined above, is fused with one or more aryl or heteroaryl groups, wherein the point of attachment is on the heterocyclyl ring, and in such instances, the number of ring members continue to designate the number of ring members in the heterocyclyl ring system. Unless otherwise specified, each instance of heterocyclyl is independently unsubstituted (an "unsubstituted heterocyclyl") or substituted (a "substituted heterocyclyl") with one or more substituents. In certain embodiments, the heterocyclyl group is an unsubstituted 3-14 membered heterocyclyl. In certain embodiments, the heterocyclyl group is a substituted 3-14 membered heterocyclyl.

In some embodiments, a heterocyclyl group is a 5-10 membered non-aromatic ring system having ring carbon atoms and 1-4 ring heteroatoms, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur ("5-10 membered heterocyclyl"). In some embodiments, a heterocyclyl group is a 5-8 membered non-aromatic ring system having ring carbon atoms and 1-4 ring heteroatoms, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur ("5-8 membered heterocyclyl"). In some embodiments, a heterocyclyl group is a 5-6 membered non-aromatic ring system having ring carbon atoms and 1-4 ring heteroatoms, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur ("5-6 membered heterocyclyl"). In some embodiments, the 5-6 membered heterocyclyl has 1-3 ring heteroatoms selected from nitrogen, oxygen, and sulfur. In some embodiments, the 5-6 membered heterocyclyl has 1-2 ring heteroatoms selected from nitrogen, oxygen, and sulfur. In some embodiments, the 5-6 membered heterocyclyl has 1 ring heteroatom selected from nitrogen, oxygen, and sulfur. Each possibility represents a separate embodiment of the present invention.

Exemplary 3-membered heterocyclyl groups containing 1 heteroatom include, without limitation, azirdinyl, oxiranyl, and thiranyl. Exemplary 4-membered heterocyclyl groups containing 1 heteroatom include, without limitation, azetidinyl, oxetanyl, and thietanyl. Exemplary 5-membered heterocyclyl groups containing 1 heteroatom include, without limitation, tetrahydrofuranyl, dihydrofuranyl, tetrahydrothiophenyl, dihydrothiophenyl, pyrrolidinyl, dihydropyrrrolyl, and pyrrolyl-2,5-dione. Exemplary 5-membered heterocyclyl groups containing 2 heteroatoms include, without limitation, dioxolanyl, oxathiolanyl and dithiolanyl. Exemplary 5-membered heterocyclyl groups containing 3 heteroatoms include, without limitation, triazolinyl, oxadiazolinyl, and thiadiazolinyl. Exemplary 6-membered heterocyclyl groups containing 1 heteroatom include, without limitation, piperidinyl, tetrahydropyranyl, dihydropyridinyl, and thianyl. Exemplary 6-membered heterocyclyl groups containing 2 heteroatoms include, without limitation, piperazinyl, morpholinyl, dithianyl, and dioxanyl. Exemplary 6-membered heterocyclyl groups containing 2 heteroatoms include, without limitation, triazinanyl. Exemplary 7-membered heterocyclyl groups containing 1 heteroatom include, without limitation, azepanyl, oxepanyl and thiepanyl. Exemplary 8-membered heterocyclyl groups containing 1 heteroatom include, without limitation, azocanyl, oxeanyl and thiocanyl. Exemplary bicyclic heterocyclyl groups include, without limitation, indolinyl, isoindolinyl, dihydrobenzofuranyl, dihydrobenzothienyl, tetrahydrobenzothienyl, tetrahydrobenzofuranyl, tetrahydroindolyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, decahydroisoquinolinyl, octahydrochromenyl, octahydroisochromenyl, decahydronaphthyridinyl, decahydro-1,8-naphthyridinyl, octahydropyrrolo[3,2-b]pyrrole, indolinyl, phthalimidyl,

naphthalimidyl, chromanyl, chromenyl, 1H-benzo[e][1,4]diazepinyl, 1,4,5,7-tetrahydropyrano[3,4-b]pyrrolyl, 5,6-dihydro-4H-furo[3,2-b]pyrrolyl, 6,7-dihydro-5H-furo[3,2-b]pyranyl, 5,7-dihydro-4H-thieno[2,3-c]pyranyl, 2,3-dihydro-1H-pyrrolo[2,3-b]pyridinyl, 2,3-dihydrofuro[2,3-b]pyridinyl, 4,5,6,7-tetrahydro-1H-pyrrolo[2,3-b]pyridinyl, 4,5,6,7-tetrahydrafuro[3,2-c]pyridinyl, 4,5,6,7-tetrahydrothieno[3,2-b]pyridinyl, 1,2,3,4-tetrahydro-1,6-naphthyridinyl, and the like.

The term “aryl” refers to a radical of a monocyclic or polycyclic (e.g., bicyclic or tricyclic) $4n+2$ aromatic ring system (e.g., having 6, 10, or 14π electrons shared in a cyclic array) having 6-14 ring carbon atoms and zero heteroatoms provided in the aromatic ring system (“ C_{6-14} aryl”). In some embodiments, an aryl group has 6 ring carbon atoms (“ C_6 aryl”; e.g., phenyl). In some embodiments, an aryl group has 10 ring carbon atoms (“ C_{10} aryl”; e.g., naphthyl such as 1-naphthyl and 2-naphthyl). In some embodiments, an aryl group has 14 ring carbon atoms (“C14 aryl”; e.g., anthracyl). “Aryl” also includes ring systems wherein the aryl ring, as defined above, is fused with one or more carbocyclyl or heterocyclyl groups wherein the radical or point of attachment is on the aryl ring, and in such instances, the number of carbon atoms continue to designate the number of carbon atoms in the aryl ring system. Unless otherwise specified, each instance of an aryl group is independently unsubstituted (an “unsubstituted aryl”) or substituted (a “substituted aryl”) with one or more substituents. In certain embodiments, the aryl group is an unsubstituted C_{6-14} aryl. In certain embodiments, the aryl group is a substituted C_{6-14} aryl.

The term “heteroaryl” refers to a radical of a 5-14 membered monocyclic or polycyclic (e.g., bicyclic, tricyclic) $4n+2$ aromatic ring system (e.g., having 6, 10, or 14π electrons shared in a cyclic array) having ring carbon atoms and 1-4 ring heteroatoms provided in the aromatic ring system, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur (“5-14 membered heteroaryl”). In heteroaryl groups that contain one or more nitrogen atoms, the point of attachment can be a carbon or nitrogen atom, as valency permits. Heteroaryl polycyclic ring systems can include one or more heteroatoms in one or both rings. “Heteroaryl” includes ring systems wherein the heteroaryl ring, as defined above, is fused with one or more carbocyclyl or heterocyclyl groups wherein the point of attachment is on the heteroaryl ring, and in such instances, the number of ring members continue to designate the number of ring members in the heteroaryl ring system. “Heteroaryl” also includes ring systems wherein the heteroaryl ring, as defined above, is fused with one or more aryl groups wherein the point of attachment is either on the aryl or heteroaryl ring, and in such instances, the number of ring members designates the number of ring members in the fused polycyclic (aryl/heteroaryl) ring system. Polycyclic heteroaryl groups wherein one ring does not contain a heteroatom (e.g., indolyl, quinolinyl, carbazolyl, and the like) the point of attachment can be on either ring, i.e., either the ring bearing a heteroatom (e.g., 2-indolyl) or the ring that does not contain a heteroatom (e.g., 5-indolyl).

In some embodiments, a heteroaryl group is a 5-10 membered aromatic ring system having ring carbon atoms and 1-4 ring heteroatoms provided in the aromatic ring system, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur (“5-10 membered heteroaryl”). In some embodiments, a heteroaryl group is a 5-8 membered aromatic ring system having ring carbon atoms and 1-4 ring heteroatoms provided in the aromatic ring

system, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur (“5-8 membered heteroaryl”). In some embodiments, a heteroaryl group is a 5-6 membered aromatic ring system having ring carbon atoms and 1-4 ring heteroatoms provided in the aromatic ring system, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur (“5-6 membered heteroaryl”). In some embodiments, the 5-6 membered heteroaryl has 1-3 ring heteroatoms selected from nitrogen, oxygen, and sulfur. In some embodiments, the 5-6 membered heteroaryl has 1-2 ring heteroatoms selected from nitrogen, oxygen, and sulfur. In some embodiments, the 5-6 membered heteroaryl has 1 ring heteroatom selected from nitrogen, oxygen, and sulfur. Unless otherwise specified, each instance of a heteroaryl group is independently unsubstituted (an “unsubstituted heteroaryl”) or substituted (a “substituted heteroaryl”) with one or more substituents. In certain embodiments, the heteroaryl group is an unsubstituted 5-14 membered heteroaryl. In certain embodiments, the heteroaryl group is a substituted 5-14 membered heteroaryl.

Exemplary 5-membered heteroaryl groups containing 1 heteroatom include, without limitation, pyrrolyl, furanyl, and thiophenyl. Exemplary 5-membered heteroaryl groups containing 2 heteroatoms include, without limitation, imidazolyl, pyrazolyl, oxazolyl, isoxazolyl, thiazolyl, and isothiazolyl. Exemplary 5-membered heteroaryl groups containing 3 heteroatoms include, without limitation, triazolyl, oxadiazolyl, and thiadiazolyl. Exemplary 5-membered heteroaryl groups containing 4 heteroatoms include, without limitation, tetrazolyl. Exemplary 6-membered heteroaryl groups containing 1 heteroatom include, without limitation, pyridinyl. Exemplary 6-membered heteroaryl groups containing 2 heteroatoms include, without limitation, pyridazinyl, pyrimidinyl, and pyrazinyl. Exemplary 6-membered heteroaryl groups containing 3 or 4 heteroatoms include, without limitation, triazinyl and tetrazinyl, respectively. Exemplary 7-membered heteroaryl groups containing 1 heteroatom include, without limitation, azepinyl, oxepinyl, and thiepinyl. Exemplary 5,6-bicyclic heteroaryl groups include, without limitation, indolyl, isoindolyl, indazolyl, benzotriazolyl, benzothiophenyl, isobenzothiophenyl, benzofuranyl, benzoisofuranyl, benzimidazolyl, benzoxazolyl, benzisoxazolyl, benzoxadiazolyl, benzthiazolyl, benzisothiazolyl, benzthiadiazolyl, indolizinyl, and purinyl. Exemplary 6,6-bicyclic heteroaryl groups include, without limitation, naphthyridinyl, pteridinyl, quinolinyl, isoquinolinyl, cinnolinyl, quinoxalinyl, phthalazinyl, and quinazolinyl. Exemplary tricyclic heteroaryl groups include, without limitation, phenanthridinyl, dibenzofuranyl, carbazolyl, acridinyl, phenothiazinyl, phenoazinyl and phenazinyl.

The term “unsaturated bond” refers to a double or triple bond. The term “unsaturated” or “partially unsaturated” refers to a moiety that includes at least one double or triple bond. The term “saturated” refers to a moiety that does not contain a double or triple bond, i.e., the moiety only contains single bonds.

Affixing the suffix “-ene” to a group indicates the group is a divalent moiety, e.g., alkylene is the divalent moiety of alkyl, alkenylene is the divalent moiety of alkenyl, alkynylene is the divalent moiety of alkynyl, heteroalkylene is the divalent moiety of heteroalkyl, heteroalkenylene is the divalent moiety of heteroalkenyl, heteroalkynylene is the divalent moiety of heteroalkynyl, carbocyclylene is the divalent moiety of carbocyclyl, heterocyclylene is the divalent moiety of heterocyclyl, arylene is the divalent moiety of aryl, and heteroarylene is the divalent moiety of heteroaryl.

A group is optionally substituted unless expressly provided otherwise. The term "optionally substituted" refers to being substituted or unsubstituted. In certain embodiments, alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl groups are optionally substituted. "Optionally substituted" refers to a group which may be substituted or unsubstituted (e.g., "substituted" or "unsubstituted" alkyl, "substituted" or "unsubstituted" alkenyl, "substituted" or "unsubstituted" alkynyl, "substituted" or "unsubstituted" heteroalkyl, "substituted" or "unsubstituted" heteroalkenyl, "substituted" or "unsubstituted" heteroalkynyl, "substituted" or "unsubstituted" carbocyclyl, "substituted" or "unsubstituted" heterocyclyl, "substituted" or "unsubstituted" aryl or "substituted" or "unsubstituted" heteroaryl group). In general, the term "substituted" means that at least one hydrogen present on a group is replaced with a permissible substituent, e.g., a substituent which upon substitution results in a stable compound, e.g., a compound which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, or other reaction. Unless otherwise indicated, a "substituted" group has a substituent at one or more substitutable positions of the group, and when more than one position in any given structure is substituted, the substituent is either the same or different at each position. The term "substituted" is contemplated to include substitution with all permissible substituents of organic compounds, and includes any of the substituents described herein that results in the formation of a stable compound. The present invention contemplates any and all such combinations in order to arrive at a stable compound. For purposes of this invention, heteroatoms such as nitrogen may have hydrogen substituents and/or any suitable substituent as described herein which satisfy the valencies of the heteroatoms and results in the formation of a stable moiety. The invention is not intended to be limited in any manner by the exemplary substituents described herein.

Exemplary carbon atom substituents include, but are not limited to, halogen, —CN, —NO₂, —N₃, —SO₂H, —SO₃H, —OH, —OR^{aa}, —ON(R^{bb})₂, —N(R^{bb})₂, 40 —N(R^{bb})₃+X⁻, —N(OR^{cc})R^{bb}, —SH, —SR^{aa}, —SSR^{cc}, —C(=O)R^{aa}, —CO₂H, —CHO, —C(OR^{cc})₂, —CO₂R^{aa}, —OC(=O)R^{aa}, —OCO₂R^{aa}, —C(=O)N(R^{bb})₂, —OC(=O)N(R^{bb})₂, —NR^{bb}C(=O)R^{aa}, —NR^{bb}CO₂R^{aa}, —NRC^{bb}(=O)N(R^{bb})₂, —C(=NR^{bb})R^{aa}, —C(=NR^{bb})₃ 45 OR^{aa}, —OC(=NR^{bb})R^{aa}, —OC(=NR^{bb})OR^{aa}, —C(=NR^{bb})N(R^{bb})₂, —OC(=NR^{bb})N(R^{bb})₂, —NR^{bb}C(=NR^{bb})N(R^{bb})₂, —C(=O)NR^{bb}SO₂R^{aa}, —NR^{bb}SO₂R^{aa}, —SO₂N(R^{bb})₂, —SO₂R^{aa}, —SO₂OR^{aa}, —OSO₂R^{aa}, —S(=O)R^{aa}, —OS(=O)R^{aa}, —Si(R^{aa})₃, —OSi(R^{aa})₃—C(=S)N(R^{bb})₂, —C(=O)SR^{aa}, —C(=S)SR^{aa}, —SC(=S) 50 SR^{aa}, —SC(=O)SR^{aa}, —OC(=O)SR^{aa}, —SC(=O)OR^{aa}, —SC(=O)R^{aa}, —P(=O)(R^{aa})₂, —P(=O)(OR^{cc})₂, —OP(=O)(R^{aa})₂, —OP(=O)(OR^{cc})₂, —P(=O)(N(R^{bb})₂)₂, —OP(=O)(N(R^{bb})₂)₂, —NR^{bb}P(=O)(R^{aa})₂, —NR^{bb}P(=O)(R^{cc})₂, 55 —NR^{bb}P(=O)(N(R^{bb})₂)₂, —P(R^{cc})₂, —P(OR^{cc})₂, —P(OR^{cc})₃+X⁻, —P(OR^{cc})₃+X⁻, —P(R^{cc})₄, —P(OR^{cc})₄, —OP(R^{cc})₂, —OP(R^{cc})₃+X⁻, —OP(OR^{cc})₂, —OP(OR^{cc})₃+X⁻, —OP(R^{cc})₄, —OP(OR^{cc})₄, —B(R^{aa})₂, —B(OR^{cc})₂, —BR^{aa}(OR^{cc}), C₁₋₁₀ alkyl, C₁₋₁₀ perhaloalkyl, C₂₋₁₀ alkenyl, C₂₋₁₀ alkynyl, heteroC₁₋₁₀ alkyl, heteroC₂₋₁₀ alkenyl, heteroC₂₋₁₀ alkynyl, C₃₋₁₀ carbocyclyl, 3-14 membered heterocyclyl, C₆₋₁₄ aryl, and 5-14 membered heteroaryl, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^d groups; wherein X is a counterion;

or two geminal hydrogens on a carbon atom are replaced with the group —O, —S, —NN(R^{bb})₂, —NNR^{bb}C(=O)R^{aa}, —NNR^{bb}C(=O)OR^{aa}, —NNR^{bb}S(=O)₂R^{aa}, —NR^{bb}, or —NOR^{cc}; each instance of R^{aa} is, independently, selected from C₁₋₁₀ alkyl, C₁₋₁₀ perhaloalkyl, C₂₋₁₀ alkenyl, C₂₋₁₀ alkynyl, heteroC₁₋₁₀ alkyl, heteroC₂₋₁₀ alkenyl, heteroC₂₋₁₀ alkynyl, C₃₋₁₀ carbocyclyl, 3-14 membered heterocyclyl, C₆₋₁₄ aryl, and 5-14 membered heteroaryl, or two R^{aa} groups are joined to form a 3-14 membered heterocyclyl or 5-14 membered heteroaryl ring, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{dd} groups; each instance of R^{bb} is, independently, selected from hydrogen, —OH, —OR^{aa}, —N(R^{cc})₂, —CN, —C(=O)R^{aa}, —C(=O)N(R^{cc})₂, —CO₂R^{aa}, —SO₂R^{aa}, —C(=NR^{cc})OR^{aa}, —C(=NR^{cc})N(R^{cc})₂, —SO₂N(R^o)₂, —SO₂R^{cc}, —SO₂OR^{cc}, —SOR^{aa}, —C(=S)N(R^{cc})₂, —C(=O)SR^{cc}, —C(=S)SR^{cc}, —P(=O)(R^{aa})₂, —P(=O)(N(R^{cc})₂)₂, C₁₋₁₀ alkyl, C₁₋₁₀ perhaloalkyl, C₂₋₁₀ alkenyl, C₂₋₁₀ alkynyl, heteroC₁₋₁₀ alkyl, heteroC₂₋₁₀ alkenyl, heteroC₂₋₁₀ alkynyl, C₃₋₁₀ carbocyclyl, 3-14 membered heterocyclyl, C₆₋₁₄ aryl, and 5-14 membered heteroaryl, or two R^{bb} groups are joined to form a 3-14 membered heterocyclyl or 5-14 membered heteroaryl ring, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{dd} groups; wherein X⁻ is a counterion; each instance of R^{cc} is, independently, selected from hydrogen, C₁₋₁₀ alkyl, C₁₋₁₀ perhaloalkyl, C₂₋₁₀ alkenyl, C₂₋₁₀ alkynyl, heteroC₁₋₁₀ alkyl, heteroC₂₋₁₀ alkenyl, heteroC₂₋₁₀ alkynyl, C₃₋₁₀ carbocyclyl, 3-14 membered heterocyclyl, C₆₋₁₄ aryl, and 5-14 membered heteroaryl, or two R^{cc} groups are joined to form a 3-14 membered heterocyclyl or 5-14 membered heteroaryl ring, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{dd} groups; each instance of R^{dd} is, independently, selected from halogen, —CN, —NO₂, —N₃, —SO₂H, —SO₃H, —OH, —OR^{ee}, —ON(R^f)₂, —N(R^f)₂, —N(R^f)₃+X⁻, —N(OR^{ee})R^f, —SH, —SR^{ee}, —SSR^{ee}, —C(=O)R^{ee}, —CO₂H, —CO₂R^{ee}, —OC(=O)R^{ee}, —OCO₂R^{ee}, —C(=O)N(R^f)₂, —OC(=O)N(R^f)₂, —NR^{ee}C(=O)R^{ee}, —NR^{ee}CO₂R^{ee}, —NR^{ee}C(=O)N(R^f)₂, —C(=NR^{ee})OR^{ee}, —OC(=NR^{ee})R^{ee}, —OC(=NR^f)OR^{ee}, —C(=NR^f)N(R^f)₂, —OC(=NR^f)N(R^f)₂, —NR^fC(=NR^f)N(R^f)₂, —NR^fSO₂R^{ee}, —SO₂N(R^f)₂, —SO₂R^{ee}, —SO₂OR^{ee}, —OSO₂R^{ee}, —S(=O)R^{ee}, —Si(R^{ee})₃, —OSi(R^{ee})₃, —C(=S)N(R^f)₂, —C(=O)SR^{ee}, —C(=S)SR^{ee}, —SC(=S)SR^{ee}, —P(=O)(OR^{ee})₂, —P(=O)(R^{ee})₂, —OP(=O)(R^{ee})₂, —OP(=O)(OR^{ee})₂, —OP(=O)(OR^{ee})₂, —C₁₋₆ alkyl, C₁₋₆ perhaloalkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, heteroC₁₋₆ alkyl, heteroC₂₋₆ alkenyl, heteroC₂₋₆ alkynyl, C₃₋₁₀ carbocyclyl, 3-10 membered heterocyclyl, C₆₋₁₀ aryl, 5-10 membered heteroaryl, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{gg} groups, or two

geminal R^{dd} substituents can be joined to form =O or =S; wherein X⁻ is a counterion; each instance of R^{ee} is, independently, selected from C₁₋₆ alkyl, C₁₋₆ perhaloalkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, heteroC₁₋₆ alkyl, heteroC₂₋₆ alkenyl, heteroC₂₋₆ alkynyl, C₃₋₁₀ carbocyclyl, C₆₋₁₀ aryl, 3-10 membered heterocyclyl, and 3-10 membered heteroaryl, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{gg} groups; each instance of R^f is, independently, selected from hydrogen, C₁₋₆ alkyl, C₁₋₆ perhaloalkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, heteroC₁₋₆ alkyl, heteroC₂₋₆ alkenyl, heteroC₂₋₆ alkynyl, C₃₋₁₀ carbocyclyl, 3-10 membered heterocyclyl, C₆₋₁₀ aryl and 5-10 membered heteroaryl, or two R^f groups are joined to form a 3-10 membered heterocyclyl or 5-10 membered heteroaryl ring, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{gg} groups; and each instance of R^{gg} is, independently, halogen, —CN, —NO₂, —N₃, —SO₂H, —SO₃H, —OH, —OC₁₋₆ alkyl, —ON(C₁₋₆ alkyl)₂, —N(C₁₋₆ alkyl)₂, —N(C₁₋₆ alkyl)₃X⁻, —NH(C₁₋₆ alkyl)₂X⁻, —NH2(C₁₋₆ alkyl)X⁻, —NH₃⁺X⁻, —N(OC₁₋₆ alkyl)(C₁₋₆ alkyl), —N(OH)(C₁₋₆ alkyl), —NH(OH), —SH, —SC₁₋₆ alkyl, —SS(C₁₋₆ alkyl), —C(=O)(C₁₋₆ alkyl), —CO₂H, —CO₂(C₁₋₆ alkyl), —OC(=O)(C₁₋₆ alkyl), —OCO₂(C₁₋₆ alkyl), —C(=O)NH₂, —C(=O)N(C₁₋₆ alkyl)₂, —OC(=O)NH(C₁₋₆ alkyl), —NHC(=O)(C₁₋₆ alkyl), —N(C₁₋₆ alkyl)C(=O)(C₁₋₆ alkyl), —NHCO₂(C₁₋₆ alkyl), —NHC(=O)N(C₁₋₆ alkyl)₂, —NHC(=O)H(C₁₋₆ alkyl), —NHC(=O)NH₂, —C(=NH)O(C₁₋₆ alkyl), —OC(=NH)(C₁₋₆ alkyl), —OC(=NH), OC₁₋₆ alkyl, —C(=NH)N(C₁₋₆ alkyl)₂, —C(=NH)NH(C₁₋₆ alkyl), —C(=NH)NH₂, —OC(=NH)N(C₁₋₆ alkyl)₂, —OC(NH)NH(C₁₋₆ alkyl), —OC(NH)NH₂, —NHC(NH)N(C₁₋₆ alkyl)₂, —NHC(=NH)NH₂, —NHSO₂(C₁₋₆ alkyl), —SO₂N(C₁₋₆ alkyl)₂, —SO₂NH(C₁₋₆ alkyl), —SO₂NH₂, —SO₂C₁₋₆ alkyl, —SO₂OC₁₋₆ alkyl, —OSO₂C₁₋₆ alkyl, —SOC₁₋₆ alkyl, —Si(C₁₋₆ alkyl)₃, —OSi(C₁₋₆ alkyl)₃, —C(=S)N(C₁₋₆ alkyl)₂, C(=S)NH(C₁₋₆ alkyl), C(=S)NH₂, —C(=O)S(C₁₋₆ alkyl), —C(=S)SC₁₋₆ alkyl, —SC(=S)SC₁₋₆ alkyl, —P(=O)(OC₁₋₆ alkyl)₂, —P(=O)(C₁₋₆ alkyl)₂, —OP(=O)(C₁₋₆ alkyl)₂, —OP(=O)(OC₁₋₆ alkyl)₂, C₁₋₆ alkyl, C₁₋₆ perhaloalkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, heteroC₁₋₆ alkyl, heteroC₂₋₆ alkenyl, heteroC₂₋₆ alkynyl, C₃₋₁₀ carbocyclyl, C₆₋₁₀ aryl, 3-10 membered heterocyclyl, 5-10 membered heteroaryl; or two geminal R⁹⁹ substituents can be joined to form =O or =S; wherein X⁻ is a counterion.

The term "halo" or "halogen" refers to fluorine (fluoro, —F), chlorine (chloro, —Cl), bromine (bromo, —Br), or iodine (iodo, —I).

The term "hydroxyl" or "hydroxy" refers to the group —OH. The term "substituted hydroxyl" or "substituted hydroxyl," by extension, refers to a hydroxyl group wherein the oxygen atom directly attached to the parent molecule is substituted with a group other than hydrogen, and includes groups selected from —OR', —ON(R^{bb})₂, —OC(=O)SR^{aa}, —OC(=O)R^{aa}, —OCO₂R^{aa}, —OC(=O)N(R^{bb})₂, —OC(=NR^{bb})R^{aa}, —OC(=NR^{bb})OR^{aa}, —OC(=NR^{bb})N(R^{bb})₂, —OS(=O)R^{aa}, —OSO₂R', —OSi(R^{aa})₃, —OP(R^{cc})₂, —OP(R^{cc})³X⁻, —OP(OR^{cc})₂, —OP(OR^{cc})³X⁻,

—OP(=O)(R^{aa}), —OP(=O)(OR^{cc})₂, and —OP(=O)(N(R^{bb}))₂, wherein X⁻, R^{aa}, R^{bb}, and R^{cc} are as defined herein.

The term "amino" refers to the group —NH₂. The term "substituted amino," by extension, refers to a monosubstituted amino, a disubstituted amino, or a trisubstituted amino. In certain embodiments, the "substituted amino" is a mono-substituted amino or a disubstituted amino group.

The term "monosubstituted amino" refers to an amino group wherein the nitrogen atom directly attached to the parent molecule is substituted with one hydrogen and one group other than hydrogen, and includes groups selected from —NH(R^{bb}), —NHC(=O)R^{aa}, —NHCO₂R^{aa}, —NHC(=O)N(R^{bb})₂, —NHC(=NR^{bb})N(R^{bb})₂, —NHSO₂R', —NHP(=O)(OR^{cc})₂, and —NHP(=O)(N(R^{bb})₂)₂, wherein R^{aa}, R^{bb} and R' are as defined herein, and wherein R^{bb} of the group —NH(R^{bb}) is not hydrogen.

The term "disubstituted amino" refers to an amino group wherein the nitrogen atom directly attached to the parent molecule is substituted with two groups other than hydrogen, and includes groups selected from —N(R^{bb})₂, —NR^{bb}C(=O)R^{aa}, —R^{bb}CO₂R^{aa}, —NR^{bb}C(=O)N(R^{bb})₂, —NR^{bb}C(=NR^{bb})N(R^{bb})₂, —NR^{bb}SO₂R^{aa}, —NR^{bb}P(=O)(ORC)₂, and —NR^{bb}P(=O)(N(R^{bb})₂)₂, wherein R^{aa}, R^{bb}, and R^{cc} are as defined herein, with the proviso that the nitrogen atom directly attached to the parent molecule is not substituted with hydrogen.

The term "trisubstituted amino" refers to an amino group wherein the nitrogen atom directly attached to the parent molecule is substituted with three groups, and includes groups selected from —N(R^{bb})₃ and —N(R^{bb})₃X⁻, wherein R^{bb} and X are as defined herein.

The term "sulfonyl" refers to a group selected from —SO₂N(R^{bb})₂, —SO₂R^{aa}, and —SO₂OR^{aa}, wherein R^{aa} and R^{bb} are as defined herein.

The term "sulfinyl" refers to the group —S(=O)R^{aa}, wherein R^{aa} is as defined herein.

The term "acyl" refers to a group having the general formula —C(=O)R^{X1}, —C(=O)OR^{X1}, —C(=O)—O—C(=O)R^{X1}, —C(=O)SR^{X1}, —C(=O)N(R^{X1})₂, —C(=S)R^{X1}, —C(=S)N(R^{X1})₂, and —C(=S)S(R^{X1}), —C(=NR^{X1})R^{X1}, —C(=NR^{X1})OR^{X1}, —C(=NR^{X1})SR^{X1}, and —C(=NR^{X1})N(R^{X1})₂, wherein R^{X1} is hydrogen; halogen; substituted or unsubstituted hydroxyl; substituted or unsubstituted thiol; substituted or unsubstituted amino; substituted or unsubstituted acyl, cyclic or acyclic, substituted or unsubstituted, branched or unbranched aliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched heteroaliphatic; cyclic or acyclic, substituted or unsubstituted, branched or unbranched alkyl; cyclic or acyclic, substituted or unsubstituted, branched or unbranched alkenyl; substituted or unsubstituted alkynyl; substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, aliphaticoxo, heteroaliphaticoxo, alkyloxy, heteroalkyloxy, aryloxy, heteroaryloxy, aliphaticethioxy, heteroaliphaticethioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroarylthioxy, mono- or di-aliphaticamino, mono- or di-heteroaliphaticamino, mono- or di-alkylamino, mono- or di-heteroalkylamino, mono- or di-arylamino, or mono- or di-heteroarylamino; or two R^{X1} groups taken together form a 5- to 6-membered heterocyclic ring. Exemplary acyl groups include aldehydes (—CHO), carboxylic acids (—CO₂H), ketones, acyl halides, esters, amides, imines, carbonates, carbamates, and ureas. Acyl substituents include, but are not limited to, any of the substituents described herein, that result in the formation of a stable moiety (e.g., aliphatic, alkyl, alkenyl, alkynyl, heteroaliphatic, heterocyclic, aryl, heteroaryl, acyl, oxo, imino, thioxo, cyano, isocyano, amino, azido, nitro,

hydroxyl, thiol, halo, aliphaticamino, heteroaliphaticamino, alkylamino, heteroalkylamino, arylamino, heteroaryl amino, alkylaryl, arylalkyl, aliphaticoxy, heteroaliphaticoxy, alkylloxy, heteroalkyloxy, aryloxy, heteroaryloxy, aliphaticthioxy, heteroaliphaticthioxy, alkylthioxy, heteroalkylthioxy, arylthioxy, heteroarylthioxy, acyloxy, and the like, each of which may or may not be further substituted).

The term "carbonyl" refers to a group wherein the carbon directly attached to the parent molecule is sp^2 hybridized, and is substituted with an oxygen, nitrogen or sulfur atom, e.g., a group selected from ketones ($-C(=O)R^{aa}$), carboxylic acids ($-CO_2H$), aldehydes ($-CHO$), esters ($-CO_2R^{aa}$, $-C(=O)SR^{aa}$, $-C(=S)SR^{aa}$), amides ($-C(=O)N(R^{bb})_2$, $-C(=O)NR^{bb}SO_2R^{aa}$, $-C(=S)N(R^{bb})_2$), and imines ($-C(=NR^{bb})R^{aa}$, $-C(=NR^{bb})OR^{aa}$), $-C(=NR^{bb})N(R^{bb})_2$), wherein R^{aa} and R^{bb} are as defined herein.

Nitrogen atoms can be substituted or unsubstituted as valency permits, and include primary, secondary, tertiary, and quaternary nitrogen atoms. Exemplary nitrogen atom substituents include, but are not limited to, hydrogen, $-OH$, $-OR^{aa}$, $-N(R'')_2$, $-CN$, $-C(=O)R^{aa}$, $-C(=O)N(R)_2$, $-CO_2R^{aa}$, $-SO_2R^{aa}$, $-C(=NR^{bb})R^{aa}$, $-C(=NR^{cc})OR^{aa}$, $-C(=NR^{cc})N(R^{cc})_2$, $-SO_2N(R^{cc})_2$, $-SO_2R^{cc}$, $-SO_2OR^{cc}$, $-SOR^{aa}$, $-C(=S)N(R^{cc})_2$, $-C(=O)SR^{cc}$, $-C(=S)SR^{cc}$, $-P(=O)(OR^{cc})_2$, $-P(=O)(R^{aa})_2$, $-P(=O)N(R^{cc})_2$, C_{1-10} alkyl, C_{1-10} perhaloalkyl, C_{2-10} alkenyl, C_{2-10} alkynyl, hetero C_{1-10} alkyl, hetero C_{2-10} alkenyl, hetero C_{2-10} alkynyl, C_{3-10} carbocyclyl, 3-14 membered heterocyclcyl, C_{6-14} aryl, and 5-14 membered heteroaryl, or two R^{cc} groups attached to an N atom are joined to form a 3-14 membered heterocyclcyl or 5-14 membered heteroaryl ring, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclcyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{dd} groups, and wherein R^{aa} , R^{bb} , R^{cc} and R^{dd} are as defined above.

In certain embodiments, the substituent present on the nitrogen atom is an nitrogen protecting group (also referred to herein as an "amino protecting group"). Nitrogen protecting groups include, but are not limited to, $-OH$, $-OR^{aa}$, $-N(R^{cc})_2$, $-C(=O)R^{aa}$, $-C(=O)N(R^{cc})_2$, $-CO_2R^{aa}$, $-SO_2R^{aa}$, $-C(=NR^{cc})R^{aa}$, $-C(=NR^{cc})OR^{aa}$, $-C(=NR^{cc})N(R^{cc})_2$, $-SO_2N(R^{cc})_2$, $-SO_2R^{cc}$, $-SO_2OR^{cc}$, $-SOR^{aa}$, $-C(=S)N(R^{cc})_2$, $-C(=O)SR^{cc}$, $-C(=S)SR^{cc}$, C_{1-10} alkyl (e.g., aralkyl, heteroaralkyl), C_{2-10} alkenyl, C_{2-10} alkynyl, hetero C_{1-10} alkyl, hetero C_{2-10} alkenyl, hetero C_{2-10} alkynyl, C_{3-10} carbocyclyl, 3-14 membered heterocyclcyl, C_{6-14} aryl, and 5-14 membered heteroaryl groups, wherein each alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carbocyclyl, heterocyclcyl, aralkyl, aryl, and heteroaryl is independently substituted with 0, 1, 2, 3, 4, or 5 R^{dd} groups, and wherein R^{aa} , R^{bb} , R^{cc} and R^{dd} are as defined herein. Nitrogen protecting groups are well known in the art and include those described in detail in *Protecting Groups in Organic Synthesis*, T. W. Greene and P. G. M. Wuts, 3rd edition, John Wiley & Sons, 1999, incorporated herein by reference.

For example, nitrogen protecting groups such as amide groups (e.g., $-C(=O)R^{aa}$) include, but are not limited to, formamide, acetamide, chloroacetamide, trichloroacetamide, trifluoroacetamide, phenylacetamide, 3-phenylpropanamide, picolinamide, 3-pyridylcarboxamide, N-benzoylphenylalanyl derivative, benzamide, p-phenylbenzamide, o-nitophenylacetamide, o-nitrophenoxycetamide, acetoacetamide, (N^t-dithiobenzoyloxyacetylaminoo)acetamide, 3-(p-hydroxyphenyl)propanamide, 3-(o-nitrophenyl)propana-

5 mide, 2-methyl-2-(o-nitrophenoxy)propanamide, 2-methyl-2-(o-phenylazophenoxy)propanamide, 4-chlorobutanamide, 3-methyl-3-nitrobutanamide, o-nitrocinnamide, N-acetylmethionine derivative, o-nitrobenzamide and o-(benzoyloxymethyl)benzamide.

Nitrogen protecting groups such as carbamate groups (e.g., $-C(=O)OR^{aa}$) include, but are not limited to, methyl carbamate, ethyl carbamate, 9-fluorenylmethyl carbamate (Fmoc), 9-(2-sulfo)fluorenylmethyl carbamate, 9-(2,7-dibromo)fluorenylmethyl carbamate, 2,7-di-t-butyl-[9-(10,10-dioxo-10,10,10,10-tetrahydrothioxanthyl)]methyl carbamate (DBD-Tmoc), 4-methoxyphenacyl carbamate (Phenoc), 2,2,2-trichloroethyl carbamate (Troc), 2-trimethylsilylethyl carbamate (Teoc), 2-phenylethyl carbamate (Adhoc), 1,1-dimethyl-2-haloethyl carbamate, 1,1-dimethyl-2,2-dibromoethyl carbamate (DB-t-BOC), 1,1-dimethyl-2,2,2-trichloroethyl carbamate (TCBOC), 1-methyl-1-(4-biphenyl)ethyl carbamate (Bpoc), 1-(3,5-di-t-butylphenyl)-1-methylethyl carbamate (t-Bumeoc), 2-(2'- and 4'-pyridyl)ethyl carbamate (Pyoc), 2-(N,N-dicyclohexylcarboxamido)ethyl carbamate, t-butyl carbamate (BOC or Boc), 1-adamantyl carbamate (Adoc), vinyl carbamate (Voc), allyl carbamate (Alloc), 1-isopropylallyl carbamate (Ipaoc), cinnamyl carbamate (Coc), 4-nitrocinnamyl carbamate (Noc), 8-quinolyl carbamate, N-hydroxypiperidinyl carbamate, alkyldithio carbamate, benzyl carbamate (Cbz), p-methoxybenzyl carbamate (Moz), p-nitrobenzyl carbamate, p-bromobenzyl carbamate, p-chlorobenzyl carbamate, 2,4-dichlorobenzyl carbamate, 4-methylsulfinylbenzyl carbamate (Msz), 9-anthrylmethyl carbamate, diphenylmethyl carbamate, 2-methylthioethyl carbamate, 2-methylsulfonylethyl carbamate, 2-(p-toluenesulfonyl)ethyl carbamate, [2-(1,3-dithianyl)]methyl carbamate (Dmoc), 4-methylthiophenyl carbamate (Mtpc), 2,4-dimethylthiophenyl carbamate (Bmpc), 2-phosphonioethyl carbamate (Peoc), 2-tri phenylphosphonioisopropyl carbamate (Ppoc), 1,1-dimethyl-2-cyanoethyl carbamate, m-chloro-p-acyloxybenzyl carbamate, p-(dihydroxyboryl)benzyl carbamate, 5-benzisoxazolylmethyl carbamate, 2-(trifluoromethyl)-6-chromonylmethyl carbamate (Troc), m-nitrophenyl carbamate, 3,5-dimethoxybenzyl carbamate, o-nitrobenzyl carbamate, 3,4-dimethoxy-6-nitrobenzyl carbamate, phenyl(o-nitrophenyl)methyl carbamate, t-amyl carbamate, S-benzyl thiocarbamate, p-cyanobenzyl carbamate, cyclobutyl carbamate, cyclohexyl carbamate, cyclopentyl carbamate, cyclopropylmethyl carbamate, p-decyloxybenzyl carbamate, 2,2-dimethoxyacetylvinyl carbamate, o-(N,N-dimethylcarboxamido)benzyl carbamate, 1,1-dimethyl-3-(N,N-dimethylcarboxamido)propyl carbamate, 1,1-dimethylpropynyl carbamate, di(2-pyridyl)methyl carbamate, 2-furanylmethyl carbamate, 2-iodoethyl carbamate, isoborynl carbamate, isobutyl carbamate, isonicotinyl carbamate, p-(p'-methoxyphenylazo)benzyl carbamate, 1-methylcyclobutyl carbamate, 1-methylcyclohexyl carbamate, 1-methyl-1-cyclopropylmethyl carbamate, 1-methyl-1-(3,5-dimethoxyphenyl)ethyl carbamate, 1-methyl-1-(p-phenylazophenyl)ethyl carbamate, 1-methyl-1-phenylethyl carbamate, 1-methyl-1-(4-pyridyl)ethyl carbamate, phenyl carbamate, p-(phenylazo)benzyl carbamate, 2,4,6-tri-t-butylphenyl carbamate, 4-(trimethylammonium)benzyl carbamate, and 2,4,6-trimethylbenzyl carbamate.

Nitrogen protecting groups such as sulfonamide groups (e.g., $-S(=O)_2R^{aa}$) include, but are not limited to, p-toluenesulfonamide (Ts), benzenesulfonamide, 2,3,6-trimethyl-4-methoxybenzenesulfonamide (Mtr), 2,4,6-trimethoxybenzenesulfonamide (Mtbs), 2,6-dimethyl-4-

methoxybenzenesulfonamide (Pme), 2,3,5,6-tetramethyl-4-methoxybenzenesulfonamide (Mte), 4-methoxybenzenesulfonamide (Mbs), 2,4,6-trimethylbenzenesulfonamide (Mts), 2,6-dimethoxy-4-methylbenzenesulfonamide (iMds), 2,2,5,7,8-pentamethylchroman-6-sulfonamide (Pmc), methanesulfonamide (Ms), (3-trimethylsilylethanesulfonamide (SES), 9-anthracenesulfonamide, 4-(4',8'-dimethoxynaphthylmethyl)benzenesulfonamide (DNMBS), benzylsulfonamide, trifluoromethylsulfonamide, and phenacylsulfonamide.

Other nitrogen protecting groups include, but are not limited to, phenothiazinyl-(10)-acyl derivative, N⁺-p-toluenesulfonylaminoacyl derivative, N⁺-phenylaminothioacyl derivative, N-benzoylphenylalanyl derivative, N-acetylmethionine derivative, 4,5-diphenyl-3-oxazolin-2-one, N-phthalimide, N-dithiasuccinimide (Dts), N-2,3-diphenylmaleimide, N-2,5-dimethylpyrrole, N-1,1,4,4-tetramethyldisilylazacyclopentane adduct (STABASE), 5-substituted 1,3-dimethyl-1,3,5-triazacyclohexan-2-one, 5-substituted 1,3-dibenzyl-1,3,5-triazacyclohexan-2-one, 1-substituted 3,5-dinitro-4-pyridone, N-methylamine, N-allylamine, N-[2-(trimethylsilyl)ethoxy]methylamine (SEM), N-3-acetoxypropylamine, N-(1-isopropyl-4-nitro-2-oxo-3-pyroline-3-yl)amine, quaternary ammonium salts, N-benzylamine, N-di(4-methoxyphenyl)methylamine, N-5-dibenzosuberylamine, N-triphenylmethylamine (Tr), N-[4-methoxyphenyl]diphenylmethylamine (MMTr), N-9-phenylfluorenylamine (PhF), N-2,7-dichloro-9-fluorenylmethyleneamine, N-ferrocenylmethylamine (Fcm), N-2-picolylamino N⁺-oxide, N-1,1-dimethylthiomethyleneamine, N-benzylideneamine, N-p-methoxybenzylideneamine, N-diphenylmethyleneamine, N-[(2-pyridyl)mesityl]methyleneamine, N-(N,N'-dimethylaminomethylene)amine, N,N'-isopropylidenediamine, N-p-nitrobenzylideneamine, N-salicylideneamine, N-5-chlorosalicylideneamine, N-(5-chloro-2-hydroxyphenyl)phenylmethyleneamine, N-cyclohexylideneamine, N-(5,5-dimethyl-3-oxo-1-cyclohexenyl)amine, N-borane derivative, N-diphenylborinic acid derivative, N-[phenyl(pentaacylchromium- or tungsten)acyl]amine, N-copper chelate, N-zinc chelate, N-nitroamine, N-nitrosoamine, amine N-oxide, diphenylphosphinamide (Dpp), dimethylthiophosphinamide (Mpt), diphenylthiophosphinamide (Ppt), dialkyl phosphoramidates, dibenzyl phosphoramidate, diphenyl phosphoramidate, benzenesulfenamide, o-nitrobenzenesulfenamide (Nps), 2,4-dinitrobenzenesulfenamide, pentachlorobenzenesulfenamide, 2-nitro-4-methoxybenzenesulfenamide, triphenylmethylsulfenamide, and 3-nitropyridinesulfenamide (Npys).

In certain embodiments, the substituent present on an oxygen atom is an oxygen protecting group (also referred to herein as an "hydroxyl protecting group"). Oxygen protecting groups include, but are not limited to, —R^{aa}, —N(R^{bb})₂, —C(=O)SR^{aa}, —C(=O)R^{aa}, —CO₂R^{aa}, —C(=O)N(R^{bb})₂, —C(=NR^{bb})R^{aa}, —C(=NR^{bb})OR^{aa}, —C(=NR^{bb})N(R^{bb})₂, —S(=O)R^{aa}, —SO₂R^{aa}, —Si(R^{aa})₃, —P(R^{cc})₂, —P(R^{cc})₃X⁻, —P(OR^{cc})₂, —P(OR^{cc})₃X⁻, —P(=O)(R^{aa})₂, —P(=O)(OR^{cc})₂, and —P(=O)(N(R^{bb})₂)₂, wherein X⁻, R^{aa}, R^{bb}, and R^{cc} are as defined herein. Oxygen protecting groups are well known in the art and include those described in detail in *Protecting Groups in Organic Synthesis*, T. W. Greene and P. G. M. Wuts, 3rd edition, John Wiley & Sons, 1999, incorporated herein by reference.

Exemplary oxygen protecting groups include, but are not limited to, methyl, methoxymethyl (MOM), methylthiomethyl (MTM), t-butylthiomethyl, (phenyldimethylsilyl)methoxymethyl (SMOM), benzyloxymethyl (BOM),

p-methoxybenzyloxymethyl (PMBM), (4-methoxyphenoxy)methyl (p-AOM), guaiacolmethyl (GUM), t-butoxymethyl, 4-pentyloxymethyl (POM), siloxymethyl, 2-methoxyethoxymethyl (MEM), 2,2,2-trichloroethoxymethyl, bis(2-chloroethoxy)methyl, 2-(trimethylsilyl)ethoxymethyl (SEMORE), tetrahydropyranyl (THP), 3-bromotetrahydropyranyl, tetrahydrothiopyran, 1-methoxycyclohexyl, 4-methoxytetrahydropyranyl (MTHP), 4-methoxytetrahydrothiopyran, 4-methoxytetrahydrothiopyran S,S-dioxide, 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (CTMP), 1,4-dioxan-2-yl, tetrahydrofuran, tetrahydrothiofuran, 2,3,3a,4,5,6,7,7a-octahydro-7,8,8-trimethyl-4,7-methanobenzofuran-2-yl, 1-ethoxyethyl, 1-(2-chloroethoxy)ethyl, 1-methyl-1-methoxyethyl, 1-methyl-1-benzyloxyethyl, 1-methyl-1-benzyloxy-2-fluoroethyl, 2,2,2-trichloroethyl, 2-trimethylsilyl-ethyl, 2-(phenylselenyl)ethyl, t-butyl, allyl, p-chlorophenyl, p-methoxyphenyl, 2,4-dinitrophenyl, benzyl (Bn), p-methoxybenzyl, 3,4-dimethoxybenzyl, o-nitrobenzyl, p-nitrobenzyl, p-halobenzyl, 2,6-dichlorobenzyl, p-cyanobenzyl, p-phenylbenzyl, 2-picolyl, 4-picolyl, 3-methyl-2-picolyl N-oxide, diphenylmethyl, p,p'-dinitrobenzhydryl, 5-dibenzosuberyl, triphenylmethyl, α -naphthylidiphenylmethyl, p-methoxyphenyldiphenylmethyl, di(p-methoxyphenyl)methyl, tri(p-methoxyphenyl)methyl, 4-(4'-bromophenacyloxyphenyl)diphenylmethyl, 4,4',4"-tris(4,5-dichlorophthalimidophenyl)methyl, 4,4',4"-tris(levulinoyloxyphenyl)methyl, 4,4',4"-tris(benzoxyloxyphenyl)methyl, 3-(imidazol-1-yl)bis(4,4"-dimethoxyphenyl)methyl, 1,1-bis(4-methoxyphenyl)-1'-pyrenylmethyl, 9-anthryl, 9-(9-phenyl)xanthenyl, 9-(9-phenyl-10-oxo)anthryl, 1,3-benzodithiolan-2-yl, benzisothiazolyl S,S-dioxide, trimethylsilyl (TMS), triethylsilyl (TES), triisopropylsilyl (TIPS), dimethylisopropylsilyl (IPDMS), diethylisopropylsilyl (DEIPS), dimethylhexylsilyl, t-butyldimethylsilyl (TBDMS), t-butyldiphenylsilyl (TBDPS), tribenzylsilyl, tri-p-xylylsilyl, triphenylsilyl, diphenylmethylsilyl (DPMS), t-butylmethoxyphenylsilyl (TBMPs), formate, benzoylformate, acetate, chloroacetate, dichloroacetate, trichloroacetate, trifluoroacetate, methoxyacetate, triphenylmethoxyacetate, phenoxyacetate, p-chlorophenoxyacetate, 3-phenylpropionate, 4-oxopentanoate (levulinate), 4,4-(ethylenedithio)pentanoate (levulinoyldithioacetal), pivaloate, adamantoate, crotonate, 4-methoxycrotonate, benzoate, p-phenylbenzoate, 2,4,6-trimethylbenzoate (mesitoate), methyl carbonate, 9-fluorenylmethyl carbonate (Fmoc), ethyl carbonate, 2,2,2-trichloroethyl carbonate (Troc), 2-(trimethylsilyl)ethyl carbonate (TMSEC), 2-(phenylsulfonyl)ethyl carbonate (Psec), 2-(triphenylphosphonio)ethyl carbonate (Peoc), isobutyl carbonate, vinyl carbonate, allyl carbonate, t-butyl carbonate (BOC or Boc), p-nitrophenyl carbonate, benzyl carbonate, p-methoxybenzyl carbonate, 3,4-dimethoxybenzyl carbonate, o-nitrobenzyl carbonate, p-nitrobenzyl carbonate, S-benzyl thiocarbonate, 4-ethoxy-1-naphthyl carbonate, methyl dithiocarbonate, 2-iodobenzoate, 4-azidobutyrate, 4-nitro-4-methylpentanoate, o-(dibromomethyl)benzoate, 2-formylbenzenesulfonate, 2-(methylthiomethoxy)ethyl, 4-(methylthiomethoxy)butyrate, 2-(methylthiomethoxy)benzoate, 2,6-dichloro-4-(1,1,3,3-tetramethylbutyl)phenoxyacetate, 2,4-bis(1,1-dimethylpropyl)phenoxyacetate, chlorodiphenylacetate, isobutyrate, monosuccinate, (E)-2-methyl-2-butenoate, o-(methoxyacetyl)benzoate, α -naphthoate, nitrate, alkyl N,N,N',N'-tetramethylphosphorodiimidate, alkyl N-phenylcarbamate, borate,

dimethylphosphinothioyl, alkyl 2,4-dinitrophenylsulfenate, sulfate, methanesulfonate (mesylate), benzylsulfonate, and tosylate (Ts).

In certain embodiments, the substituent present on a sulfur atom is a sulfur protecting group (also referred to as a “thiol protecting group”). Sulfur protecting groups include, but are not limited to, —R^{aa}, —N(R^{bb})₂, —C(=O)SR^{aa}, —C(=O)R^{aa}, —CO₂R¹, —C(=O)N(R^{bb})₂, —C(=NR^{bb})R^{aa}, —C(=NR^{bb})OR^{aa}, —C(=NR^{bb})N(R^{bb})₂, —S(=O)R^{aa}, —SO₂R¹, —Si(R^{aa})₃, —P(R^{cc})₂, —P(R^{cc})₃⁺X⁻, —P(OR^{cc})₂, —P(OR^{cc})₃⁺X⁻, —P(=O)(R^{aa})₂, —P(=O)(OR^{cc})₂, and —P(=O)(N(R^{bb})₂), wherein R^{aa}, R^{bb}, and R^{cc} are as defined herein. Sulfur protecting groups are well known in the art and include those described in detail in *Protecting Groups in Organic Synthesis*, T. W. Greene and P. G. M. Wuts, 3rd edition, John Wiley & Sons, 1999, incorporated herein by reference.

A “counterion” or “anionic counterion” is a negatively charged group associated with a positively charged group in order to maintain electronic neutrality. An anionic counterion may be monovalent (i.e., including one formal negative charge). An anionic counterion may also be multivalent (i.e., including more than one formal negative charge), such as divalent or trivalent. Exemplary counterions include halide ions (e.g., F⁻, Cl⁻, Br⁻, I⁻), NO₃⁻, ClO₄⁻, OH⁻, H₂PO₄⁻, HCO₃⁻, HSO₄⁻, sulfonate ions (e.g., methansulfonate, trifluoromethanesulfonate, p-toluenesulfonate, benzene-sulfonate, 10-camphor sulfonate, naphthalene-2-sulfonate, naphthalene-1-sulfonic acid-5-sulfonate, ethan-1-sulfonic acid-2-sulfonate, and the like), carboxylate ions (e.g., acetate, propanoate, benzoate, glycerate, lactate, tartrate, glycolate, gluconate, and the like), BF₄⁻, PF₄⁻, PF⁻, AsF₆⁻, SbF₆⁻, B[3.5-(CF₃)₂C₆H₃]I⁻, B(C₆F₅)⁻, BPh₄⁻, Al(OC(CF₃)₃)⁻, and carborane anions (e.g., CB₁₁H₁₂⁻ or (HCB₁₁Me₅Br₆)⁻). Exemplary counterions which may be multivalent include CO₃²⁻, HPO₄²⁻, PO₄³⁻, B₄O₇²⁻, SO₄²⁻, S₂O₃²⁻, carboxylate anions (e.g., tartrate, citrate, fumarate, maleate, malate, malonate, gluconate, succinate, glutarate, adipate, pimelate, suberate, azelate, sebacate, salicylate, phthalates, aspartate, glutamate, and the like), and carbo-ranes.

As used herein, use of the phrase “at least one instance” refers to 1, 2, 3, 4, or more instances, but also encompasses a range, e.g., for example, from 1 to 4, from 1 to 3, from 1 to 2, from 2 to 4, from 2 to 3, or from 3 to 4 instances, inclusive.

These and other exemplary substituents are described in more detail throughout. The invention is not intended to be limited in any manner by the above exemplary listing of substituents.

As used herein, the term “salt” refers to any and all salts, and encompasses pharmaceutically acceptable salts.

The term “pharmaceutically acceptable salt” refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response, and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, Berge et al. describe pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 1977, 66, 1-19, incorporated herein by reference. Pharmaceutically acceptable salts of the compounds of this invention include those derived from suitable inorganic and organic acids and bases. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids, such as hydrochloric acid, hydrobromic acid, phosphoric acid, sul-

furic acid, and perchloric acid or with organic acids, such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid, or malonic acid or by using other methods known in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Salts derived from appropriate bases include alkali metal, alkaline earth metal, ammonium, and N⁺(C₁₋₄ alkyl)₄⁻ salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate, and aryl sulfonate.

EXAMPLES

30 Formulation and Performance of High Purity PEG Lipids

Batches of PEG lipids of varying purities were studied. Small scale lots (gram-scale) of highly purified Compound (I) (compounds of Formula (I), HO-PEG-Stearate) were made, and in moving towards the production of Compound (B)/Compound (I) LNPs, a survey of commercial sources for the Compound (I) component indicated that two choices for sourcing were available: (a) sourcing bulk HO-PEG-Stearate from a supplier such as Sigma, or (b) requesting custom synthesis from specialty vendors such as Corden or BioVectra.

Comparison of the two choices revealed a disparity in chemical purity of HO-PEG-stearate that was dependent on the source (Table 1). Differences in chemical impact were not understood and have not been reported until now. The dramatic differences in chemical purity necessitates the demonstration of the significance of Compound (I) purity in formulation and process development, and in bioperformance.

TABLE 1

Chemical purity of HO-PEG-stearate (Compound (I))		
Source	Lot No.	Lipid Purity Parent Peak %
Synthesized	Lot No. 1	97.7%, n = 3
	Lot No. 2	96.2%, n = 3
	Lot No. 3	97.0%, n = 3
	Lot No. 4	90.3%, n = 3
	Lot No. 5	97.8%, n = 3
	Lot No. 6	98.0%, n = 3
	Lot No. 7	71.2%, n = 3
	Lot No. 8	97.8%, n = 3
Corden	Lot No. 9	98.5%, n = 3
	Lot No. 10	86.6%, n = 3
BioVectra	PEG-40-Stearate	43.8%, n = 3

To determine the recommended chemical purity for Compound (I), the formulation and process development expe-

127

rience and readout in bioperformance were assessed by formulating LNPs using low, medium, and high purity Compound (I), and subjecting these formulations to in vitro and in vivo testing.

Materials and Methods

Definitions: DOSY NMR: Diffusion ordered spectroscopy Nuclear Magnetic Resonance; UPLC-CAD: Ultra performance liquid chromatography with Charge aerosol detector; BEEFI: Biophysical escape from encapsulation by fluorescence intercalation; DLS: Dynamic light scattering; PEG: polyethylene glycol; G5 eGFP and G5 hEPO: Moderna mRNA fifth generation enhanced green fluorescent protein and human erythropoietin protein.

TABLE 2A

Materials	
Material	Source
Compound (I) (lot# Lot No. 1, wbu-249, Lot No. 3)	Moderna Delivery Chemistry Group
Compound (I) (lot# Lot No. 4, Lot No. 5, Lot No. 6, Lot No. 7, Lot No. 8)	Moderna Scale-up Process Chemistry Group
Compound (I) (lot# Lot No. 9)	Corden
Compound (I) (Lot No. 10)	BioVectra
PEG-40-Stearate (Cat# P3440 SIGMA)	Sigma Aldrich
OL-66	Moderna Scale-up Process Chemistry
Compound (B) (lot# YZ-625-208)	Organix
1,2-distearyl-sn-glycero-3-phosphocholine (DSPC) (Cat# 850365)	Avanti Polar Lipids
Cholesterol (Cat# 700000)	Avanti Polar Lipids
1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene Glycol (PEG2k-DMG) (Cat# SUNBRIGHT ® GM-020)	NOF America Corporation
N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearyl-sn-glycero-3-phosphoethanolamine (PEG2k-DSPE) (Cat# SUNBRIGHT ® DSPE-020CN)	NOF America Corporation
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)	Avanti Polar Lipids
(Rhodamine-DOPE) (Cat# 810150)	
G5 eGFP and G5 hEPO	Moderna Production Team

TABLE 2B

Analytical Methods	
Test	Method/Equipment
Chemical purity	UPLC, Thermo Fisher Vanquish
Formulation Preparation	Microfluidic nanoprecipitation, NanoAssemblr, Precision Nanosystems
Particle size distribution and poly dispersity	Dynamic Light Scattering, Wyatt DynaPro Plate Reader II
Lipid Quantification	UPLC-CAD, Thermo Fisher Vanquish
mRNA Quantification	UPLC, Thermo Fisher Vanquish
Encapsulation Efficiency and BEEFI (in vitro mRNA escape assay)	Quant-it™ Ribogreen assay, Synergy BioTek Plate Reader
Heparin-Sepharose binding assay	UPLC

128

TABLE 2B-continued

Analytical Methods	
Test	Method/Equipment
PEG diffusion	DOSY NMR, Bruker 300 MHz NMR Spectrometer
in vitro uptake and expression	Hela cell incubation, live cell imaging and quantification on OPERA system
IgM binding and ApoE binding	Beads-based flow cytometry assay, BD Fortessa

Preparation and Characterization of LNP Formulations

15 Lipid nanoparticles (LNPs) comprising Compound (B) as the cationic lipid component and compounds of Formula (I) (Compound (I)) as the PEG component maintain protein expression without stimulating the immune system. With this Compound (B)/Compound (I) combination, protein expression is observed in both human hepatocytes and Kupffer cells with Compound (B)/Compound (I), and anti-PEG IgM levels and complement activation remain low (rodent).

20 25 Lipids were dissolved in ethanol to a total lipid concentration of 12.5 mM at molar ratios of 50:10:38.5:1.5 (Compound (B): DSPC: cholesterol: PEG-lipid). mRNA was diluted to 0.125 mg/mL in 6.25 mM sodium acetate buffer (pH 5). The lipid solution and mRNA solution were mixed at a ratio of 3:1 (aqueous: ethanol) on a microfluidic chip using the NanoAssemblr. Formulations were dialyzed against phosphate-buffered saline (pH 7.2) in dialysis cassettes overnight (ca. 12-18 h). Subsequently, formulations were concentrated using 100 kDa MWCO Amicon ultra 30 centrifugal filters, and syringe filtered using 0.22 µm Supor 35 membranes.

40 45 Lipid components and formulations were analyzed by many analytical techniques. Chemical purity of lipid components was determined using an ultra-performance liquid chromatography (UPLC) method on a Thermo Fisher Vanquish system. Particle size distribution diameter and polydispersity index (PDI) of particles were measured using dynamic light scattering (DLS) on a Wyatt DynaPro Plate reader II. Encapsulation efficiency and level of endosomal escape was determined by a fluorescent, plate-based assay, Quant-it™ Ribogreen assay using the Synergy BioTek plate reader. Lipid quantification and mRNA quantification of formulations were determined by ultra-performance liquid 50 55 chromatography. Surface properties of the formulations were determined by a heparin-Sepharose binding assay on an ultra-performance liquid chromatography system. PEG diffusion was measured using DOSY NMR on a 300 MHz NMR Bruker Avance III spectrometer. In vitro uptake and 55 60 expression of formulations in Hela cells was determined using live cell imaging and quantification on an OPERA microscopy system. IgM binding and ApoE binding were measured using a beads-based flow cytometry assay on a BD Fortessa. In vivo experiments were conducted by the in-house pharmacology team in accordance to IACUC protocols.

In Vivo Study of PEG Lipid Purity

The in vivo design for initial assessment of the impact of 65 Compound (I) chemical purity on expression, B cell activation, and anti-PEG IgM generation upon multi-dosing is outlined in Table 3.

TABLE 3

In vivo study design for Compound (I) chemical purity assessment						
Group	Sample (batch; purity)	# of Doses	# of Animals	Dose Level (mg/kg)	Dose Volume (mL)	Dose Concentration (mg/mL)
1	Compound (B)/PEG DMG	3	8	0.5	0.1	0.1
2	Compound (B)/Compound (I) (Lot No. 5; 98%)	3	8	0.5	0.1	0.1
3	Compound (B)/Compound (I) (BioVectra; 87%)	3	8	0.5	0.1	0.1
4	Compound (B)/Compound (I) (Sigma; 44%)	3	8	0.5	0.1	0.1
5	Compound (B)/Compound (I) (2x Sigma; 44%)	3	8	0.5	0.1	0.1
6	Compound (B)/PEG DSPE	3	8	0.5	0.1	0.1
7	1x PBS	3	3	0.5	0.1	0.1

Endpoints for each arm are:

(a) hEPO ELISA 6 h post each dose;

(b) Anti-PEG IgM 96 h post 2nd and 3rd dose;

(c) B cell activation; 3 spleens from each dose group 24 h post 3rd dose

Prediction of Storage Stability

The impact of Compound (I) chemical purity on LNP stability was interrogated by storing each formulation in a freezable buffer (e.g., 20 mM Tris 8% Sucrose) and freezing the vial in a -20° C. freezer. Particle growth assessment occurred after time zero at 1, 2 and 3 freeze thaws. In addition to particle size and PDI measurements, endpoint characterizations include % EE and PEG shedding by DOSY NMR spectroscopy.

TABLE 4

Effect of Compound (I) chemical purity on formulation stability to freeze thaw				
Group	Sample (batch; purity)	Buffer Storage	[mRNA] mg/mL	Vial fill (mL)
1	Compound (B)/PEG DMG	20 mM Tris 8% Sucrose, -20 C.	0.5	0.5
2	Compound (B)/Compound (I) (Lot No. 5; 98%)	20 mM Tris 8% Sucrose, -20 C.	0.5	0.5
3	Compound (B)/Compound (I) (BioVectra; 87%)	20 mM Tris 8% Sucrose, -20 C.	0.5	0.5
4	Compound (B)/Compound (I) (Sigma; 44%)	20 mM Tris 8% Sucrose, -20 C.	0.5	0.5
5	Compound (B)/Compound (I) (2 x Sigma; 44%)	20 mM Tris 8% Sucrose, -20 C.	0.5	0.5

Data and Discussion

As chemical purity may influence drug product performance and on drug substance (DS) and drug product (DP) manufacture, the properties of Compound (I) DS were

evaluated with respect to the following criteria: (a) the drug substance manufacturing/process experience, (b) effect of chemical purity on bioperformance. Data from development as well as the in vitro and in vivo campaigns were utilized to build a better understanding on the size of the operating window.

(a) Formulation Process Experience

The formulations containing different purity of Compound (I) did not perform the same during formulation (Table 5). Compound (I) Lot No. 7 (71% purity) did not dissolve in the stock solution and was not used further. OL-66 LNPs precipitated shortly after microfluidic mixing. Although the formulations which were successful showed similar % EE and PSD (Table 5), their actual DLS profiles indicate very different particle size distributions (FIG. 2).

nd=not determined.

TABLE 5

Formulation experience using Compound (I) of different chemical purity					
Group	Name	Lipid Purity %	post-dialysis (0.2 μm filtered)		
			Moderna	Diameter (nm)	PDI
1	86-56 Corden	99	89.7	0.16	98
2	86-56 Lot No. 5	98	92.1	0.16	97
3	86-56 BioVectra	87	94.4	0.18	98
4	86-56 Sigma	44	99.3	0.19	97
4	86-56 Lot No. 7	71	n/d	n/d	nd
5	86-66	97	nd	nd	nd

The actual amount of lipid incorporated into the LNPs was found to depend on the initial starting purity of Compound (I) (Table 6 and FIG. 3).

TABLE 6

Lipid quantification in LNPs									
	Total Lipids mg/mL	Compound (I) mol %	Compound (B) mol %	Cholesterol mol %	DSPC mol %	[mRNA] mg/mL	Calculated Lipid: mRNA wt:wt	Theoretical Lipid: mRNA wt:wt	
86-56 Corden	14.466	1.72%	49.44%	39.44%	9.40%	0.737	19.62	20.83	
86-56 Lot No. 5	12.915	1.74%	50.36%	38.54%	9.35%	0.668	19.35	20.83	
86-56 BioVectra	11.131	1.55%	50.42%	38.55%	9.48%	0.580	19.20	20.83	
86-56 Sigma	11.648	0.69%	51.00%	39.03%	9.28%	0.617	18.88	20.83	

15

All formulations have similar binding to the heparin-sepharose column (FIG. 4) which indicates that this chromatography assay is unable to detect differences in surface properties. The PEG-shedding rate from LNPs in each of the different formulations incubated in mouse serum were also found to be indistinguishable from one another by DOSY-NMR spectroscopy (FIG. 5). Although the surface characteristics prior to PEG shedding were indistinguishable from one another, once the PEG lipid was shed from the LNP, the in vitro assay measuring mRNA escape demonstrated that those formulations using higher purity Compound (I) showed greater amounts of accessible mRNA by Ribogreen detection (FIG. 6).

Because the Sigma lot of Compound (I) was very low in chemical purity, using this lot and accounting for the deficiency in purity (adjusted the amount of Sigma PEG-lipid added to match theoretical mol %) resulted in formulations with similar physiochemical characteristics (Tables 7 and 8; FIG. 7). Similar PEG shedding of LNP was observed by DOSY-NMR spectroscopy.

TABLE 7

Formulation experience using Compound (I) using highly impure Compound (I)						
Group	Name	Formulation Composition	mol %	Moderna post-dialysis (0.2 um filtered)		
				Lipid Purity %	Diameter (nm)	PDI
7	86-56 Sigma	Compound (B): DSPC: Chol: Compound (I)	50:10:38.5:1.5	44 (Double amount of PEG lipid)	91	0.16 97

TABLE 8

Lipid quantification from a formulation using highly impure Compound (I)								
	Total Lipids mg/mL	Compound (I) mol %	Compound (B) mol %	Cholesterol mol %	DSPC mol %	[mRNA] mg/mL	Calculated Lipid: mRNA wt:wt	Theoretical Lipid: mRNA wt:wt
86-56 Sigma	17.822	1.37%	50.38%	38.40%	9.85%	0.718	24.79	20.83

60

Formulations prepared for in vitro fluorescence assays were found to have similar physiochemical properties as the unlabeled Compound (I) LNPs (Table 9). As in the non-labeled DLS data, the actual profiles showed discrepancies which are not noticeable when only Z-average and PDI are reported (FIG. 8). nd=not determined.

TABLE 9

Formulation experience using fluorescent Compound (I) LNP ^s							
Group	Name	Formulation Composition	mol %	Moderna	post-dialysis (0.2 μm filtered)		
				Lipid Purity %	Diameter (nm)	PDI	% EE
65-1	86 PEG DMG	Compound (B): DSPC: Chol: Rhod-DOPE: PEG-DMG	50:10:38.4:0.1:1.5	nd	71.2	0.19	97
65-2	86-56 Corden	Compound (B): DSPC: Chol: Rhod-DOPE: Compound (I)	50:10:38.4:0.1:1.5	99	97.5	0.18	96
65-3	86-56 Lot No. 5	Compound (B): DSPC: Chol: Rhod-DOPE: Compound (I)	50:10:38.4:0.1:1.5	98	93.2	0.19	96
65-4	86-56 BioVectra	Compound (B): DSPC: Chol: Rhod-DOPE: Compound (I)	50:10:38.4:0.1:1.5	87	97.9	0.2	97
65-5	86-56 Sigma*	Compound (B): DSPC: Chol: Rhod-DOPE: Compound (I)	50:10:38.4:0.1:1.5	44	105.5	0.22	97
65-6	86-56 Sigma**	Compound (B): DSPC: Chol: Rhod-DOPE: Compound (I)	50:10:38.4:0.1:1.5	44	86.6	0.2	95
65-7	86 PEG DSPE	Compound (B): DSPC: Chol: Rhod-DOPE: PEG DSPE	50:10:38.4:0.1:1.5	nd	69.4	0.17	98

*One addition of Sigma Compound (I), ~0.6-0.7 mol % Compound (I) in final product

**Two additions of Sigma Compound (I), ~ 1.3-1.4 mol % Compound (I) in final product

(b) Effect of Chemical Purity on Bioperformance

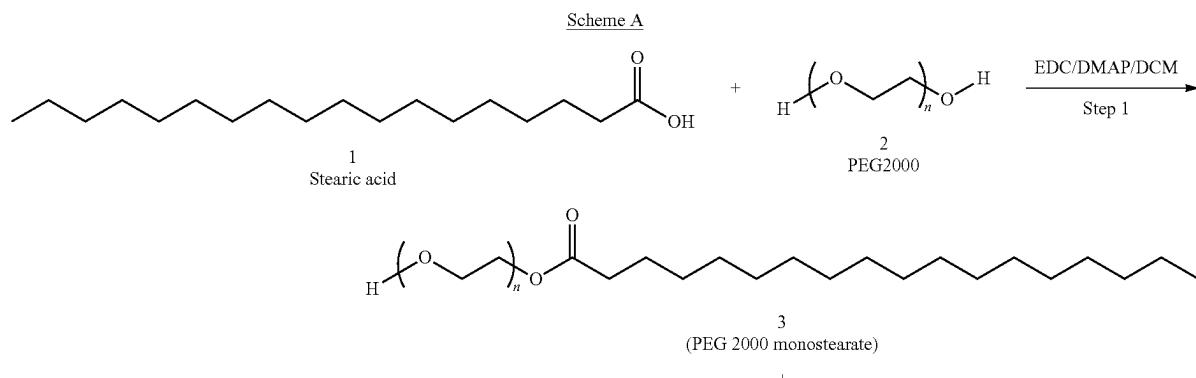
To predict the effect of chemical purity on in vivo performance, the formulations were tested in vitro and in vivo. The ApoE binding panel showed similar bind for the formulations in low IgM and high IgM containing serum. ApoE binding decreases with decreasing purity level of Compound (I) (FIG. 9). The effect on immune response was studied in IgM activation assays (FIG. 10). Formulations containing Compound (I) with a purity level of <87% have higher IgM binding, which is a clear indication that there is a purity threshold for Compound (I) at which in vivo performance will suffer. The lower ApoE binding and higher IgM binding for LNPs containing impure Compound (I) as compared to pure Compound (I) suggest purity plays an important role in influencing biologic performance.

Based on the manufacturability assessment, the acceptable range of chemical purity is estimated to be greater than or equal to 87%. The lower limit is based on the proven ability to process the formulations and minimization of immune response IgM.

Synthesis of PEG Lipids

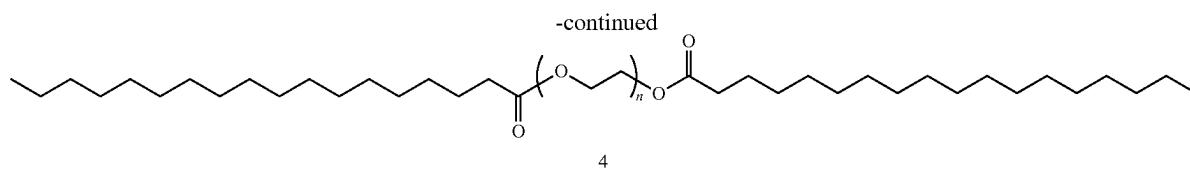
The exemplary manufacturing protocol of Compound (I) is a convergent synthesis involving the coupling reaction of Stearic acid (1) and polyethylene glycol 2000 (2), as shown in Scheme A. The synthetic process is a 1-step synthesis with 30 executed under current Good Manufacturing Practice (cGMP or GMP) conditions. The reaction involves the esterification of 1 with 2, using 4-(dimethylamino) pyridine (DMAP), N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in dichloromethane (DCM) followed by workup and purification to produce Compound (I).

The reaction is monitored using thin layer chromatography (TLC) and ultra-high pressure chromatography with charged aerosol detection (UPLC-CAD) until the vast majority of the reactants are consumed. During the purification with column chromatography, fractions are analyzed by UPLC-CAD and are combined if a purity criteria of ≥97.5 area % is met. Additional information on in-process controls is provided below.



135

136



Reaction of stearic acid (1 eq), PEG2000 (2 eq) and EDC in dichloromethane. A mixture of stearic acid (5.0 g), PEG2000 (70.3 g) and DMAP (429 mg) in DCM (125 mL) was cooled to <5° C. by ice bath. At <5° C., EDC (5.05 g) was added to the reaction mixture at <5° C. and the mixture was stirred at this temperature for 10 minutes. DCM (125 mL) was dropwise added to the reaction mixture at <5° C. over 25 minutes, the reaction mixture was allowed to warm to 20° C. and stirred overnight. The reaction solution was cooled to <5° C. by ice bath. 5% NaHCO₃ solution (125 mL, prepared by mixing 6.25 g NaHCO₃ and 118.75 g water) was dropwise added to the reaction solution at <5° C. over 15 min. The reaction mixture was allowed to warm to ambient temperature by removing ice bath. The organic layer (emulsion) was separated from aqueous layer (colorless but turbid) and washed with 5% NaHCO₃/5% NaCl solution (125 mL, prepared by mixing 6.25 g NaHCO₃, 6.25 g NaCl and 118.75 g water). The murky organic layer was separated from colorless aqueous layer (pH 8 by pH paper) and concentrated in vacuo at 35° C. to give off-white solid. The off-white solid was diluted in MeCN, aged at 35° C. and then concentrated in vacuo at 35° C. to give 80.8 g (203%) of off-white solid. The off-white solid was dissolved in a mixture of MeCN (400 mL), water (200 mL), TBME (200 mL) and n-heptane (200 mL). Clear ternary phase was formed in 30 minutes. LCMS shows that top layer contains no product and middle layer contains PEG2000 monostearate. Bottom layer contains PEG2000 and PEG2000 monostearate with a ratio of 1:1. The middle layer was concentrated in vacuo at 35° C. and then co-evaporated with MeCN

15
20
25
30
35
40
45

Reaction of stearic acid (1 eq), PEG2000 (10 eq) and EDC in dichloromethane. A mixture of stearic acid (2.13 g), PEG2000 (150 g) and DMAP (183 mg) in DCM (135 mL) was cooled to <5° C. EDC (2.15 g) was added to the reaction mixture at <5° C. and the mixture was stirred at this temperature for 10 minutes. DCM (135 mL) was dropwise added to the reaction mixture at <5° C. over 30 minutes. The reaction mixture was stirred at this temperature for 10 min and then allowed to warm to 20° C. and stirred for 16 hours. At 23° C., 1% brine solution (mixture of 2.7 g NaCl and 270 mL) was added to the reaction mixture at 23° C. over 2 min. LCMS shows no product loss into aqueous layer. The organic layer was separated from aqueous layer and washed with 1% brine solution (mixture of 2.7 g NaCl and 270 mL). The organic layer was separated and concentrated in vacuo at 35° C. to give off-white solid which was further diluted in MeCN (250 mL), aged at 35° C. for 20 min and concentrated in vacuo at 35° C. to give off-white solid (98.7 g, 582% yield). The solid was partitioned in MeCN (500 mL), 2% brine solution (10 g NaCl+H₂O 500 mL), MTBE (250 mL) and n-Heptane (250 mL) to give a clear three-layer phase separation. The middle layer was concentrated in vacuo at 35° C. to give PEG2000 monostearate (12.3 g, 72.7% yield, 93.90% UPLC-CAD purity).

FIG. 13 shows a flowchart of the exemplary synthesis/purification method.

Control of Materials

The starting materials used for synthesis of Compound (I) are shown in Table 10.

TABLE 10

Compound (I) Starting Materials		
Starting Material	Molecular Formula (Molecular Weight)	Chemical Structure
Compound 1	C ₁₈ H ₃₆ O ₂ (284.48)	<p style="text-align: center;">stearic acid</p>
Compound 2	HO—[C ₂ H ₄ O ₂] _n —C ₁₈ H ₃₅ O (2047-2484)	<p style="text-align: center;">Polyoxethylene 2000</p>

to give PEG2000 monostearate (32.51 g, 81.6% yield, 92.1% UPLC-CAD purity) as off-white solid.

65 The manufacturer's specifications for the starting materials Compound 1 (Emery Oleochemicals, Malaysia) and

Compound 2 (Clariant, Germany) are provided in Table 11. Raw materials used in the manufacture of Compound (I) are provided in Table 12 and

Table.

TABLE 11

Starting Material Specifications				
Material	Manufacturer and Address	Test	Method	Acceptance Criteria
Compound 1	Emery Oleochemicals Sdn. Bhd Telor Panglima Garang, Lot 4, Jalan Perak Kawasan Perusahaan, Telok Panglima Garang 42500 Selangor Malaysia	Appearance Color Identity Purity	Visual Visual ¹ H-NMR (CDCl ₃) UPLC-CAD LoD	Solid Off-white to white M = 284.5 ± 1.5 m/z Comparable to reference ≥98% ≤1.0%
Compound 2	Clariant Produkte GmbH Werk Gendorf Industrieparkstraße 1 84508 Burgkirchen Germany	Appearance Color Identity Purity	Visual Visual ¹ H-NMR (CDCl ₃) UPLC-CAD LoD	Solid Off-white to white M = 1982.02 ± 218.5 m/z Comparable to reference ≥98.0% ≤1.0%

TABLE 12

Reagent Specifications				
Material	Test	Method	Acceptance Criteria	
4-(Dimethylamino)-pyridine (C ₇ H ₁₀ N ₂)	Appearance Color Identity Purity	Visual Visual MS (ESI pos.) Assay	Solid Pale yellow to off-white 122.17 ± 1 u ≥99%	
N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (C ₈ H ₁₈ ClN ₃)	Appearance Color Identity Purity	Visual Visual MS (ESI pos.) Argentometric Assay	Solid Off-white to white 159.25 ± 1 u (free base form) ≥98%	
Sodium Bicarbonate (NaHCO ₃)	Appearance Color Identification Sodium carbonates Purity	Visual Visual Ph Eur. Assay	Crystalline solid White Positive pH NMT 8.6 ≥99%	
Sodium Chloride (NaCl)	Appearance Color Identity Purity	Visual Visual MS (ESI neg.) Argentometric Assay	Solid White 58.44 ± 1 u ≥99.5% Assay	

TABLE 13

Solvent Specifications				
Material	Test	Method	Acceptance Criteria	
Acetonitrile (CH ₃ CN)	Appearance Color Identity Purity	Visual Visual IR Spectrum GLC (area %)	Liquid Clear and colorless Comparable to reference standard ≥99.5% Refractive Index (n ²⁰) 1.344 ± 0.002	
	Additional Tests	Non-volatile matter (w/w)	Max 0.005% (w/w)	
Methanol (CH ₄ O)	Appearance Color Identity Purity	Visual Visual IR Spectrum GLC (area %)	Clear liquid Colorless Comparable to reference standard ≥99.9% (area %) Refractive Index (n ²⁰) 1.329 ± 0.001	

TABLE 13-continued

Solvent Specifications			
Material	Test	Method	Acceptance Criteria
Methylene Chloride (CH ₂ Cl ₂)	Appearance	Visual	Clear liquid
	Color	Visual	Colorless
	Identity	IR Spectrum	Comparable to reference standard
	Purity	GLC	≥99.5% (area %)
Methyl tert-Butyl Ether (C ₅ H ₁₂ O)	Appearance	Refractive Index (n _D ²⁰)	1.424 ± 0.001
		Visual	Clear liquid
	Color	Visual	Colorless
		IR Spectrum	Comparable to reference standard
	Identity	GLC (area %)	≥99.8% (area %)
		Water (KF)	≤0.1%
n-Heptane (C ₇ H ₁₆)	Appearance	Refractive Index (n _D ²⁰)	1.369 ± 0.002
		Peroxides	≤50 mg/L
	Color	Visual	Liquid
		Visual	Colorless
Purified Water (H ₂ O)	Identity	IR Spectrum	Comparable to reference standard
		GC	≥99%
	Assay	Water (KF)	≤0.01%
		USP, Ph. Eur.	Clear colorless liquid
Purified Water (H ₂ O)	TOC	USP, Ph. Eur.	Meets requirements
	Conductivity	USP, Ph. Eur.	Meets requirements
	Total Count	USP, Ph. Eur.	Alert Limit ≥ 5 CFU/mL Action Limit ≥ 100 CFU/mL
	Endotoxins	USP, Ph. Eur.	≤0.25 EU/mL
	Heavy Metals	USP, Ph. Eur.	NMT 0.1 ppm
	Nitrates	USP, Ph. Eur.	NMT 0.2 ppm

In-process controls for the Compound (I) manufacturing process are listed in Table 14.

testing. The HPLC-CAD method was qualified prior to release testing of the GMP Lot G0545. A number of opti-

TABLE 14

In-Process Controls for Compound (I) Manufacturing Process				
No.	In-Process Control	Method	Purpose	Specifications
1	Conversion check	TLC and CAD-UPLC	Verify sufficient conversion of Compound 1 and Compound 2 starting materials	CAD-UPLC: ≤5 area % for Compound 1 ≥40 area % for Compound (I)
2	Check of the washing solutions for NaHCO ₃	TLC	Verify absence of product in the washing solution.	Undetectable Compound (I) product in the last rinse
3	Purity check of the crude product	TLC and CAD-UPLC	Verify the purity of the crude product.	CAD-UPLC: 40-60 area % Compound (I)
4	Check of the n-Heptane wash	TLC	Verify absence of Compound (I) in the rinse	Undetectable Compound (I) in the rinse
5	Check of the aqueous wash solution	TLC	Verify only a small amount of product is present in the aqueous phase.	Undetectable Compound (I) product left in H ₂ O.
6	Purity check of the crude product	TLC and CAD-UPLC	Verify the purity of the crude product.	CAD-UPLC: 50-99 area % Compound (I)
7	Purity check of the column fractions	TLC and CAD -UPLC	Verify purity of the column fractions. (Various test pools are taken from the fractions; ones determined to be pure by TLC subjected to evaporation of solvents and analyzed by CAD-UPLC to determine purity).	CAD-UPLC: ≥95 area % Compound (I)
8	Check of the Line Clearance fractions	TLC	Verify absence of product in eluate for terminating the purification step	Absence of Compound (I) product in eluate

Summary of process optimization: (1) Small-scale familiarization pre-run for process optimization; (2) Manufacture of ~200 grams of non-GMP material for toxicological studies, a portion of which was qualified as the primary reference standard; (3) Manufacture of 270 g of GMP material.

The compounds Poly(ethylene glycol) 2000 (PEG2000, Compound 1) and Stearic acid (Compound 2) were designated as the GMP starting materials. A HPLC-CAD purity method was developed to support in-process and release

mizations were established and subsequently implemented as summarized below. Both starting materials, PEG2000 (Compound 1) and Stearic acid (Compound 2), are commercially available and were purchased from qualified vendors as provided above. The familiarization of the synthesis process resulted in the isolation of 198 grams of non-GMP material, of which 30 grams of primary reference substance was subsequently qualified.

141

The description of the GMP Batch Synthesis is shown in Figure B. Quantities of 2250 grams and 160 grams of GMP starting materials, PEG2000 (Compound 1) and Stearic acid (Compound 2), respectively, were purchased and used for the manufacture of the GMP Lot G0545. These two starting materials, plus all reagents/solvents used during the GMP step, were released according to defined specifications. Compound 1 was released using MALDI-MS for the determination of the number-average molecular weight, main average of the weight-average molecular weight as part of the release specification. Compound 2 was released using an established GC purity method as part of the release specification. Familiarization runs prior to the GMP synthesis also allowed for establishment of suitable IPC analysis points.

142

The GMP synthesis of Compound (I) was performed at the R&D scale at a GMP facility, with purification by column chromatography performed within the kilo-lab GMP facilities. The Compound (I) GMP product was isolated (Lot G0545, 270 g, 21% yield for final step) and released against the pre-defined specifications. The quality attributes for reference standard, non-GMP and GMP Compound (I) Lots are summarized and compared in Table 15, Table 16, and Table 17. RT: retention time (RRT Compound (I)=1.00); NQ: not quantified; NT: not tested.

Figure B

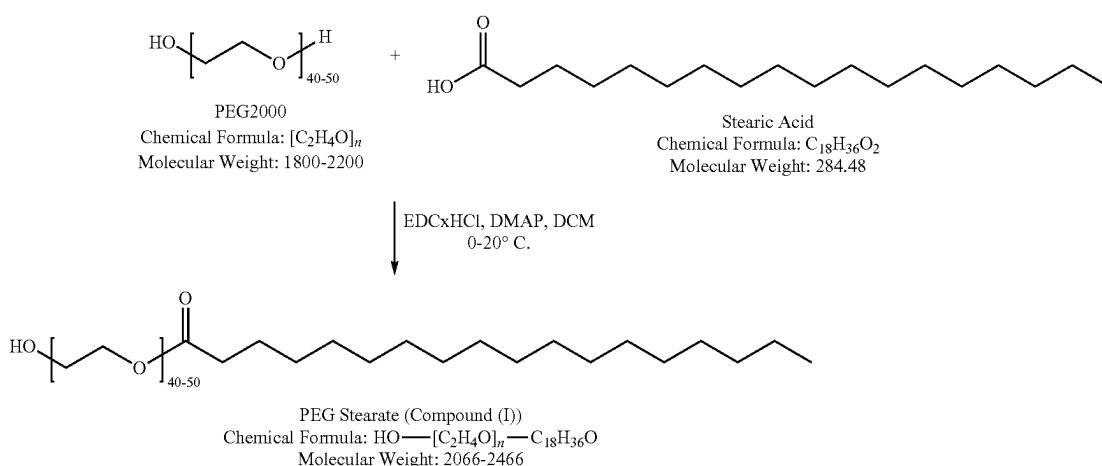


TABLE 15

Test	Method	Acceptance Criteria	Lot STD-00287 (Ref Std)
Identification	Visual	Solid	Solid
	Visual	White to off-white	White
	MS (MALDI)	Mn = 2047-2484 m/z	2300
	UPLC-CAD	RT of main peak corresponds to reference	RT of main peak corresponds to reference
	IR	Comparable to reference	Comparable to reference
	¹ H-NMR	Conforms to structure	Conforms to structure
	¹³ C-NMR	NT	NT
Purity	UPLC-CAD	≥90 area %	97.9
Assay	UPLC-CAD	90-110% w/w	97.6
Water	Karl Fischer	≤3.0% w/w	0.20
Residue on ignition	USP <281>, EP	≤0.1% w/w	0.07
	2.4.14		
Melting point	USP <741>	Report range [° C.]	54.2-54.7
Peroxides	Photometric	Report results	NQ. (<0.8 mmol/kg)
		Residual Solvents	
Acetonitrile	Headspace GC-	≤410 ppm	<100 (NQ)
MTBE	FID	≤5000 ppm	<10 (NQ)
Dichloromethane		≤600 ppm	248
n-Heptane		≤5000 ppm	<10 (NQ)
Methanol		≤3000 ppm	<100 (NQ)
Acetone		≤5000 ppm	NT

US 12,383,508 B2

143

144

TABLE 15-continued

Test	Method	Acceptance Criteria	Lot STD-00287 (Ref Std)
Elemental Impurities			
Arsenic (As)	ICP-MS	≤1.5 ppm	<0.05 (NQ)
Cadmium (Cd)		≤0.2 ppm	<0.05 (NQ)
Cobalt (Co)		≤0.5 ppm	<0.05 (NQ)
Copper (Cu)		≤30 ppm	0.05
Mercury (Hg)		≤0.3 ppm	<0.05 (NQ)
Lithium (Li)		≤25 ppm	<0.05 (NQ)
Nickel (Ni)		≤2 ppm	0.05
Lead (Pb)		≤0.5 ppm	<0.05 (NQ)
Antimony (Sb)		≤9 ppm	<0.05 (NQ)
Vanadium (V)		≤1 ppm	<0.05 (NQ)
Iron (Fe)		Report result (ppm)	0.84
Silicon (Si)		Report result (ppm)	7.23
Microbial Limits			
TAMC	USP <61>, EP	≤100 CFU/g	NT
TYMC	2.6.12	≤100 CFU/g	NT
Bacterial endotoxins	USP <85>, EP 2.6.14	≤20 EU/g	NT

TABLE 16

Test	Lot RD-01341 (Non-GMP)	Lot JLI-000099-11 (Non-GMP)	Lot G0545 (GMP)
Appearance	Solid	Solid	Solid
Color	White	White	White
Identification	2356	2322	2313
	RT of main peak corresponds to reference	RT of main peak corresponds to reference	RT of main peak corresponds to reference
	Comparable to reference	Comparable to reference	Comparable to reference
	Conforms to structure	Conforms to structure	Conforms to structure
	NT	NT	NT
Purity	97.6	95.3	98.2
Assay	97.3	95.2	99.9
Water	0.20	0.10	0.26
Residue on ignition	0.06	0.02	<0.01
Melting point	53.9-54.5	53.6-54.1	53.9-54.4
Peroxides	NQ. (<0.8 mmol/kg)	NQ (<0.8 mmol/kg)	NQ. (<0.8 mmol/kg)
Residual Solvents			
Acetonitrile	<100 (NQ)	<100 (NQ)	<100 (NQ)
MTBE	<10 (NQ)	<10 (NQ)	10
Dichloromethane	397	<100 (NQ)	<100 (NQ)
n-Heptane	<10 (NQ)	<10 (NQ)	<10 (NQ)
Methanol	<100 (NQ)	<100 (NQ)	<100 (NQ)
Acetone	NT	NT	<50 (NQ)
Elemental Impurities			
Arsenic (As)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Cadmium (Cd)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Cobalt (Co)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Lot STD-00287			
Test	Method	Acceptance Criteria	(Ref Std)
Copper (Cu)	0.06	0.12	0.11
Mercury (Hg)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Lithium (Li)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Nickel (Ni)	0.19	0.37	0.08
Lead (Pb)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Antimony (Sb)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Vanadium (V)	<0.05 (NQ)	<0.05 (NQ)	<0.05 (NQ)
Iron (Fe)	1.7	3.8	0.32
Silicon (Si)	7.0	6.1	<5.00 (NQ)
Microbial Limits		Microbial Limit	s

TABLE 16-continued

TAMC	NT	NT	<10 CFU/g
TYMC	NT	NT	<10 CFU/g
Bacterial endotoxins	NT	NT	<10 EU/g

TABLE 17

Batch Analyses Data for Compound (I) Related Impurities				
RRT	Area % by CAD			
Compound (I) is RRT 1.00	Lot STD-00287 (Ref Std)	Lot RD-01341 (Non-GMP)	Lot JLI-000099-11 (Non-GMP)	Lot G0545 (GMP)
0.55-0.56	0.43	0.40	2.35	0.14
0.90	0.13	0.13	0.17	ND
0.93	0.29	0.29	0.29	0.30
0.96-0.97	0.33	0.32	0.26	0.26
1.04	0.23	0.23	0.34	0.39
1.08-1.09	0.45	0.43	0.40	0.47
1.35	ND	ND	0.36	ND
1.37-1.38	0.22	0.61	0.52	0.22
Total	2.1	2.4	4.7	1.78

Elucidation of Structure and Other Characteristics

The structure of Compound (I) was elucidated using the following analytical procedures: Infrared spectroscopy, ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy, and MALDI-TOF mass spectrometry. All analytical and spectroscopic data were collected for the reference standard.

The Infrared (IR) absorbance spectrum of Compound (I) was recorded on a Perkin Elmer spectrometer by Corden Pharma LLC. The spectrum is shown in FIG. 12A with bands assignment listed in Table 18.

TABLE 18

Bands Assignment for IR	
Wavenumber (cm ⁻¹)	Functional group
1059.9, 1103.1	C—O—C (PEG)
1240.7	C—O—C (Ester)
1466.3	CH ₂

The ¹H and ¹³C-NMR spectra of Compound (I) were acquired on a Bruker DPX 300 at a frequency of 300 MHz (¹H) and 75 MHz (¹³C) and measured in deuterated chloroform (CDCl₃). The free induction decays (FIDs) were processed using Fourier Transform application software Bruker Topspin 1.3/ICON-NMR. The ¹H and ¹³C-NMR spectra of Compound (I) (FIG. 12B and FIG. 12C, respectively) along with peak assignments (Table 19 and Table 20, respectively) for Compound (I) are provided below. The observed ¹H and ¹³C-NMR spectral assignments are consistent with the structure of Compound (I), according to hydrogen and carbon numbering.

TABLE 19

¹ H NMR Assignments		
d [ppm]	Description	Assignment
4.3	Triplet, 2H	—OCH ₂ —CH ₂ —O—(CO)—CH ₂ —CH ₂ — (proton assignment A)
3.73	Multiplet, 2 H	—O—CH ₂ —CH ₂ —O—(CO)—CH ₂ —CH ₂ — (proton assignment B)
3.65	Multiplet, 184 H	—CH ₂ —Glycol (proton assignment C-all PEG units apart from A and B)
2.25	Triplet, 2H	—O—(CO)—CH ₂ —CH ₂ — (proton assignment D)
1.62	Multiplet, 2H	—O—(CO)—CH ₂ —CH ₂ — (proton assignment E)
1.23	Singlet, 28 H	—CH ₂ —stearic acid (proton assignment F-all stearic acid CH ₂ units apart from D and E)
0.8	Triplet, 3H	—CH ₃ (proton assignment G)

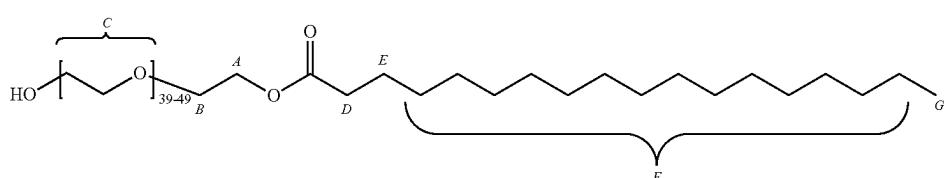
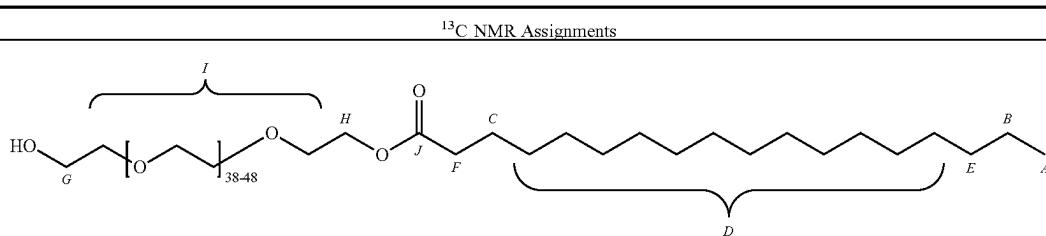


TABLE 20



d [ppm]	Description	Assignment
14.59	Single Peak	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_3$ (carbon assignment A)
23.80	Single Peak	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\overline{\text{CH}}_3$ (carbon assignment B)
26.10	Single Peak	$-\text{O}-\text{CH}_2-\overline{\text{CH}}_2-\text{O}-(\text{CO})-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ (carbon assignment C)
30.2-30.9	Multiple Peaks	$-\overline{\text{CH}}_2$ -stearic acid (carbon assignment D: all stearic acid methylene carbons apart from A, B, C, E and F)
33.13	Single Peak	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_3$ (carbon assignment E)
35.04	Single Peak	$-\text{O}-\text{CH}_2-\overline{\text{CH}}_2-\text{O}-(\text{CO})-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ (carbon assignment F)
62.30	Single Peak	$\text{HO}-\overline{\text{CH}}_2-\text{CH}_2-\text{O}-\text{CH}_2-\overline{\text{CH}}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$ (carbon assignment G)
64.62	Single Peak	$-\overline{\text{O}}-\text{CH}_2-\text{CH}_2-\text{O}-(\text{CO})-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ (carbon assignment H)
70.2-73.8	Multiple Peaks	$-\overline{\text{CH}}_2-\text{O}-\text{glycol}$ (carbon assignment I: all glycol methyleneoxy carbons apart from G and H)
175.28	Single Peak	$-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-(\text{CO})-\text{CH}_2-\text{CH}_2-\text{CH}_2-$ (carbon assignment J)

MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) mass spectrometry for Compound (I) was performed using an Applied Biosystems Voyager-DE PRO Biospectrometry Work Station coupled with a Delayed Extraction laser-desorption mass spectrometry. Compound (I) gave a major series of singly charged pseudo molecular ion clusters observed between m/z 1710 and 2730, centered at approximately m/z 2377. The measured mass of 2300 m/z (Mn) is in good agreement within the specified molecular weight range of 2047-2484. See FIG. 12D.

EQUIVALENTS AND SCOPE

In the claims articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

Furthermore, the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Where elements are presented as lists, e.g., in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements and/or features, certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements and/or features. For purposes of simplicity, those embodiments have not been specifically set forth in *haec verba* herein.

It is also noted that the terms "comprising" and "containing" are intended to be open and permits the inclusion of additional elements or steps. Where ranges are given, end-points are included. Furthermore, unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or sub-range within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

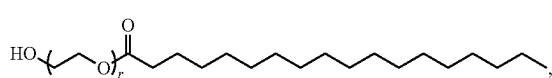
This application refers to various issued patents, published patent applications, journal articles, and other publications, all of which are incorporated herein by reference. If there is a conflict between any of the incorporated references and the instant specification, the specification shall control.

In addition, any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Because such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the invention can be excluded from any claim, for any reason, whether or not related to the existence of prior art.

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation many equivalents to the specific embodiments described herein. The scope of the present embodiments described herein is not intended to be limited to the above Description, but rather is as set forth in the appended claims. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

What is claimed is:

1. A lipid nanoparticle (LNP) comprising a plurality of PEG lipids of Formula (I):

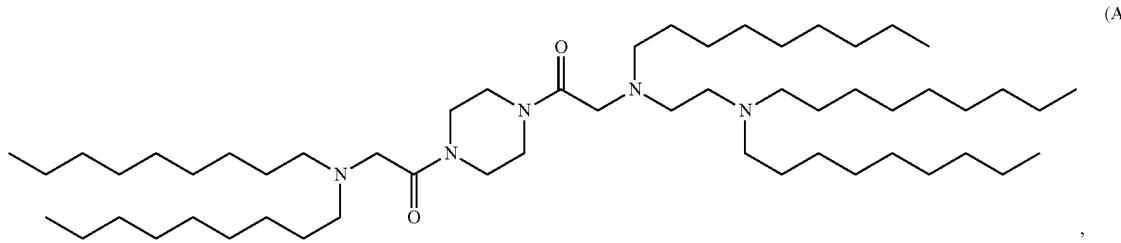


or salts thereof;

149

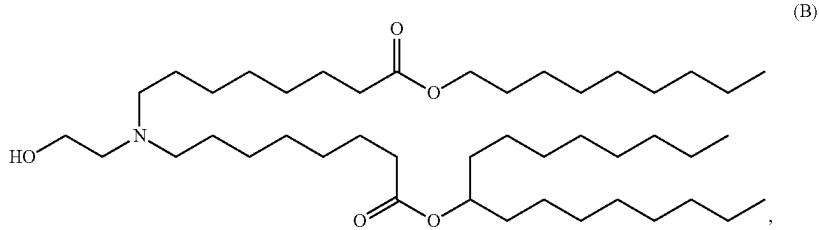
wherein r is independently an integer from 35-55, inclusive; and
wherein the plurality of PEG lipids has a purity of 87% or greater with respect to the PEG lipid component of the LNP.

2. The LNP of claim 1 further comprising an ionizable amino lipid.
3. The LNP of claim 1 further comprising a helper lipid.
4. The LNP of claim 1 further comprising a structural lipid.
5. The LNP of claim 1 comprising the plurality of PEG lipids, an ionizable amino lipid, a helper lipid, and a structural lipid.
6. The lipid nanoparticle (LNP) of claim 1 comprising Compound (A):



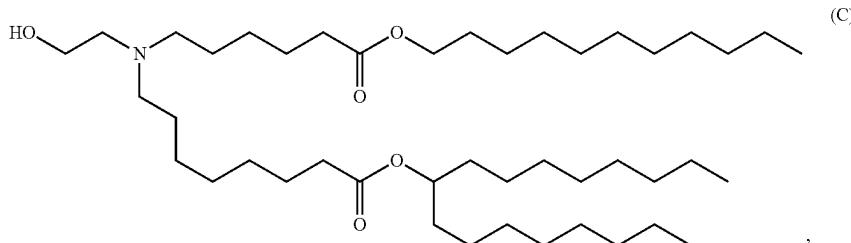
the plurality of PEG lipids of Formula (I), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol.

7. The lipid nanoparticle (LNP) of claim 1 comprising Compound (B):



the plurality of PEG lipids of Formula (I), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol.

8. The lipid nanoparticle (LNP) of claim 1 comprising Compound (C):



the plurality of PEG lipids of Formula (I), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol.

9. The LNP of claim 1 further comprising a therapeutic agent.

150

10. The LNP of claim 9, wherein the therapeutic agent is a nucleic acid.

11. The LNP of claim 9, wherein the therapeutic agent is an mRNA.

5 12. The LNP of claim 1, wherein the LNP formulation exhibits lower IgM binding as compared to an LNP formulation comprising a plurality of PEG lipids with a purity of less than 87%.

10 13. The LNP of claim 1, wherein the LNP formulation exhibits higher ApoE binding as compared to an LNP formulation comprising a plurality of PEG lipids with a purity of less than 87%.

14. The LNP of claim 1, wherein the LNP formulation is less sensitive to accelerated blood clearance (ABC) as

compared to an LNP formulation comprising a plurality of PEG lipids with a purity of less than 87%.

30 15. A method of delivering a therapeutic agent to a subject, the method comprising administering to the subject

45 an LNP of claim 1; wherein the LNP further comprises the therapeutic agent, and wherein the LNP encapsulates the therapeutic agent.

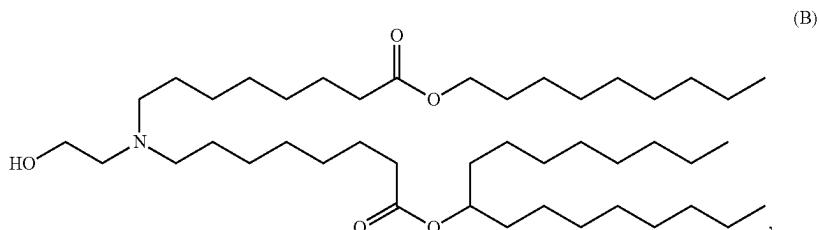
16. The LNP of claim 1, wherein r is independently an integer from 40-50, inclusive.

65 17. The LNP of claim 1, wherein r is independently an integer from 42-48, inclusive.

18. The LNP of claim 1, wherein r is 45.

151

- 19.** The LNP of claim **1**, wherein the purity of the plurality of PEG lipids is greater than 90%.
- 20.** The LNP of claim **1**, wherein the purity of the plurality of PEG lipids is greater than 95%.
- 21.** The LNP of claim **1**, wherein the purity of the plurality of PEG lipids is greater than 98%. **5**
- 22.** The LNP of claim **1**, wherein the PEG lipid component of the LNP is substantially free of impurities.
- 23.** The LNP of claim **5** comprising a molar ratio of 0.15-15% of the plurality of PEG lipids of Formula (I), **10** 25-65% of the ionizable amino lipid, 30-50% of the structural lipid, and 10-40% of the helper lipid.
- 24.** The LNP of claim **2**, wherein the ionizable amino lipid is Compound (B):



or a pharmaceutically acceptable salt thereof.

- 25.** The LNP of claim **7** comprising a molar ratio of 40% or less of Compound (B), 0.15-15% of the plurality of PEG **30** lipids of Formula (I), 20-40% of DSPC, and 30-50% of cholesterol.

- 26.** The LNP of claim **7** comprising a molar ratio of 25-35% of Compound (B), 1-2% of the plurality of PEG lipids of Formula (I), 25-35% of DSPC, and 35-42% of **35** cholesterol.

- 27.** The LNP of **7** further comprising a therapeutic agent.

- 28.** The LNP of claim **27**, wherein the therapeutic agent is an mRNA.

- 29.** A method of delivering a therapeutic agent to a **40** subject, the method comprising administering to the subject an LNP of claim **7**; wherein the LNP further comprises the therapeutic agent, and wherein the LNP encapsulates the therapeutic agent.

* * * * *