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Engineering Liposomes and Nanoparticles for Biological Targeting

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Abstract Our ability to engineer nanomaterials for biological and medical applications is continuously increasing, and nanomaterial designs are becoming more and more complex. One very good example of this is the drug delivery field where nanoparticle systems can be used to deliver drugs specifically to diseased tissue. In the early days, the design of the nanoparticles was relatively simple, but today we can surface functionalize and manipulate material properties to target diseased tissue and build highly complex drug release mechanisms into our designs. One of the most promising strategies in drug delivery is to use ligands that target overexpressed or selectively expressed receptors on the surface of diseased cells. To utilize this approach, it is necessary to control the chemistry involved in surface functionalization of nanoparticles and construct highly specific functionalities that can be used as attachment points for a diverse range of targeting ligands such as antibodies, peptides, carbohydrates and vitamins. In this review we provide an overview and a critical evaluation of the many strategies that have been developed for surface functionalization of nanoparticles and furthermore provide an overview of how these methods have been used in drug delivery systems.

Keywords Biological targeting · Drug delivery · Functionalization · Liposome · Nanoparticle

Abbreviations

CAC	Critical aggregation concentration
ConA	Concanavalin A
DCC	*N,N′*-dicyclohexylcarbodiimide
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine

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DOPS	1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine
DPPE	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DSPC	1,2-Distearoyl-*sn*-glycero-3-phosphocholine
DSPE	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELAM	Endothelial-leukocyte adhesion molecule-1
EPR	Enhanced permeation and retention
GPI	Glycophosphatidylinositol
HER2	Human epidermal growth factor receceptor 2
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin G
LAMP	Lysosome-associated membrane protein
MAb	Monoclonal antibodies
MESNA	Mercaptoethanesulfonate
mon2C5	Monoclonal antinucleosome antibody 2C5
mon2G4	Monoclonal antimyosin antibody 2G4
NBD	7-Nitro-1,2,3-benzoxadiazole
NHS	*N*-hydroxysuccinimide
PDP	*N*-(3′-(pyridyldithio)propionoyl
PE	Phosphatidylethanolamine
PEG	Poly(ethylene glycol)
PL	Phospholipids
PLA2	Phosholipase A2
*p*NP	*p*-Nitrophenylcarbonyl
QCM	Quartz crystal microbalance
RES	Reticuloendothelial system
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
TfR	Transferrin receptor
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WGA	Wheat germ agglutinin

Contents

1 Introduction

The pharmaceutical industry has successfully developed numerous drugs for the treatment of cancer, but it remains one of the world's most devastating diseases with more than 10 million new incidences every year [105]. One of the major obstacles to current treatments is inadequate delivery of the therapeutics to the tumor site, which leads to severe side effects [8]. There is therefore increasing demand for delivery systems that transport the drug specifically to the diseased tissue and improve the therapeutic index of the encapsulated drug. Nanoparticle systems have been investigated as drug delivery systems for several decades, and strategies are becoming increasingly complex to fulfill the growing requirement for treating diseases. The nanoparticle drug delivery systems offer new treatment regimes for a large variety of different diseases; however, due to the relatively high costs of many of the utilized materials, they have mainly found applications for treatment of cancer. Of the many classes of nanocarrier systems that are currently under investigation, liposomes remain one of the most successful. Liposomes were first proposed as drug delivery vehicles by Gregoriadis et al. [54] in 1974. Early liposomal formulations suffered from rapid clearance by phagocytic cells of the reticuloendothelial system (RES). This issue was solved by coating the liposomes with polymers, particularly poly(ethylene glycol) (PEG), which suppress protein absorption and opsonization of the liposomes [139]. These *Stealth*® or *sterically stabilized* liposomes were found to accumulate in tumors and inflammatory tissue by passive diffusion because of the leaky vasculature and the lack of an effective lymphatic drainage present in such tissues [82, 87]. This phenomenon is commonly referred to as the enhanced permeation and retention effect (EPR effect). Despite the enhanced accumulation in cancerous or inflammatory tissue as a result of the EPR effect, there is strong motivation for improving the accumulation by

coating the outer liposomal membrane with ligands targeted towards overexpressed or selectively expressed receptors on diseased cells. *Active targeting* with ligands, such as peptides, carbohydrates, glycoproteins, antibodies or fragments thereof, has been utilized to selectively deliver drugs to the desired site of action by increasing the nanocarrier accumulation. Targeted liposomes/nanoparticles are superior compared to drug immunoconjugates since only few targeting ligands are needed to deliver several thousands drug molecules. Furthermore, liposomes are highly biocompatible and can protect encapsulated drugs from premature degradation in the blood stream. However, surface functionalization of liposomes or nanoparticles with targeting ligands are not trivial even though multiple reports have already utilized the targeting strategy. One of the largely overlooked problems when attaching targeting ligands is that reactions that normally work well in solution may proceed very slowly on a surface, and care should be taken to select the chemistry that is suitable for the desired synthetic manipulation. In particular, in many studies there is no evaluation of the success of the functionalization, which is highly problematic as many of the utilized chemistries are far from quantitative.

The aim of this review is to summarize the recent advances in the field of nanoparticle functionalization. The focus is on surface functionalization of liposomes, but the discussed chemistry is equally relevant for other nanoparticle constructs. We will discuss the many new and highly specific conjugation methods that have been developed in recent years to functionalize liposomes and give examples of how different classes of targeting ligands have been attached to liposomes and used to target diseased tissue. We will furthermore briefly discuss the membrane anchors that are employed, which is an overlooked problem in many studies.

2 Surface Functionalization of Liposomes

Three methods are commonly used to functionalize liposomes with targeting ligands. Small targeting ligands, such as vitamins [44, 77, 117], saccharides [25, 33, 122, 149] and small peptides [37, 39], are often covalently attached to a hydrophobic anchor (e.g., a lipid) in organic solvent and purified. The functionalized lipid can thereafter be mixed with natural lipids and hydrated to form liposomes. This approach is only possible when working with smaller ligands and is particularly useful when the ligand comes in relatively large quantities (due to the purification step). A major advantage of the approach is the complete control of the amount of ligands per liposome since the initially added amount can be varied in a controlled manner. However, approximately 50% of the added functionalized lipids will be oriented towards the interior of the liposome, thus, not interacting with the outer environment.

Another way to introduce specific ligands at the outer liposomal membrane is the *post-insertion* approach [62], which is useful for expensive ligands. In this

approach the ligands are typically covalently coupled to preformed lipid–PEG micelles (e.g., DSPE–PEG), which have functionality in the distal end of the PEG that allows coupling to the ligand. Alternatively, synthesized and purified ligand–lipid moieties made by the strategy discussed above can also be used. Succeeding, incubation of the micelles with preformed liposomes allows the DSPE–PEG–ligand conjugates to transfer from the micelles into the outer liposomal membrane in a temperature- and time-dependent manner, if the process is thermodynamically favored. This approach has been used to functionalize liposomes with antibodies [6, 13, 36, 61, 62, 100], peptides [95, 114, 135] and proteins [24]. A major advantage of this approach is that the loading of the liposomes is decoupled from the insertion of the ligands, which allows for optimization of both parameters. Targeting liposomes prepared by the *post-insertion* approach have been shown to have the same in vitro drug leakage rates, cell association profiles and therapeutic efficacies compared to liposomes made by other approaches [6, 61, 95]. However, the amount of ligands inserted into the liposome membrane must be quantified when using this approach.

Functionalization of liposomes with targeting ligands can also be carried out by *post-functionalization*, e.g., performing the conjugation directly on the preformed liposomes with anchors exposing specific functionalities in their respective head groups. This method is primarily used with larger and complex ligands, such as proteins and antibodies or fragments thereof. One should realize that the reactions often do not go to completion, and the degree of functionality should always be quantified. A large number of different anchors, e.g., fatty acids, phospholipids and sterols, have been used. The effect and the properties of these anchors will be discussed in the end of this review.

Ideally, surface coupling reactions should be simple, fast, efficient, reproducible and result in bonds that are non-toxic and non-immunogenic. Furthermore, reaction conditions for surface functionalization should be mild in order to retain the biological activity of the targeting ligands. A wide range of coupling methods has successfully been developed during the last 25 years, resulting in a broad variety of possible methods to functionalize liposomes. Early coupling methods are generally characterized by unspecific surface functionalization resulting in moderate yields, whereas the modern approaches enable site-specific functionalization in high yields. Each coupling reaction used to covalently attach ligands to the liposome surface will be described separately in order to highlight the advantages and disadvantages of the various methods. To limit the scope of this review, only surface functionalization of liposomes is discussed; however, the surface chemistry applies for the majority of other nanoparticle-based drug delivery systems.

2.1 Coupling of Ligands to Amine-Modified Liposomes

One of the earliest developed methods to covalently couple ligands to the liposome surface is based on amine functionalized liposomes. Torchilin et al. [124, 125]

described the use of two homobifunctional crosslinkers (Fig. 1a), glutaraldehyde (**1**) and dimethyl suberimidate (**2**) (Fig. 2), for amine–amine crosslinking. Addition of either **1** or **2** to DPPE-containing liposomes resulted in up to 70% imine or amidine formation, respectively, at the liposome surface. Incubation of these liposomes with rabbit anticanine cardiac myosin antibodies at 4°C in aqueous buffer resulted in 60% conversion [125] without loss of the binding capacity of the antigen.

The major advantage of this surface functionalization approach is the fact that it is based on naturally occurring lipids, which can be used without prior derivatization. However, the use of homobifunctional crosslinkers can result in uncontrollable homopolymerization of ligands or liposomes during the crosslinking reaction, which can lead to liposome aggregation. Furthermore, since multiple amine functionalities are usually present in antibodies, a random attachment can be

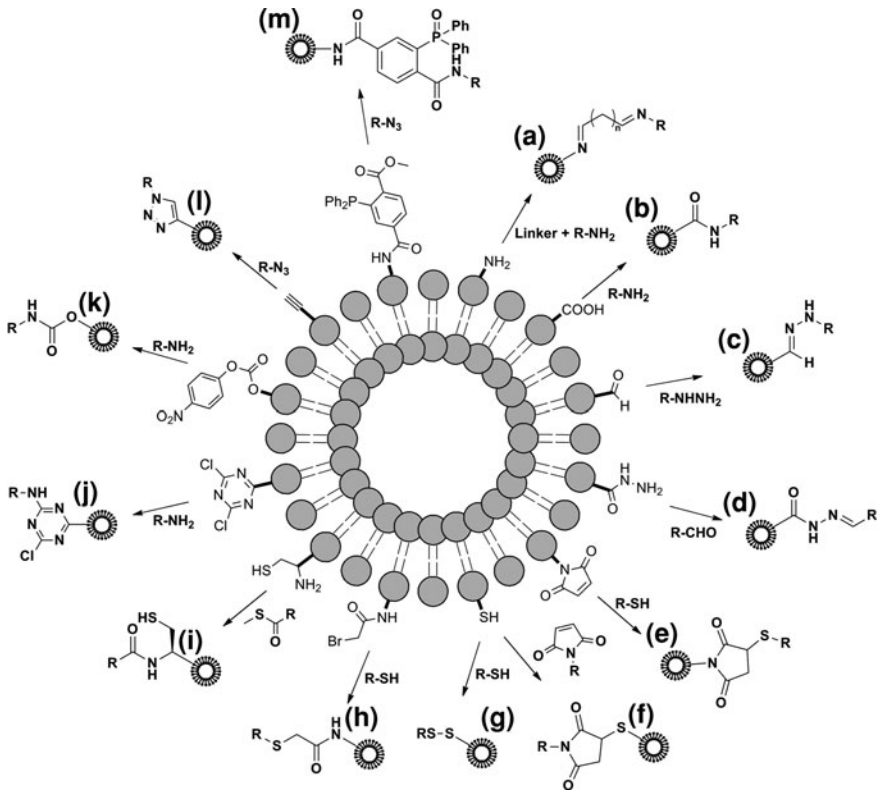

Fig. 1 Schematic illustration of the various coupling methods that have been developed in order to post-functionalize liposomes. **a** Amine functionalization, **b** carboxylic acid functionalization, **c** aldehyde functionalization, **d** hydrazine functionalization, **e** maleimide functionalization, **f** thiol functionalization, **g** thiol functionalization (disulfide bond formation), **h** bromoacetyl functionalization, **i** cysteine functionalization, **j** cyanur functionalization, **k***p*-nitrophenylcarbonyl functionalization, **l** alkyne functionalization, **m** triphosphine functionalization

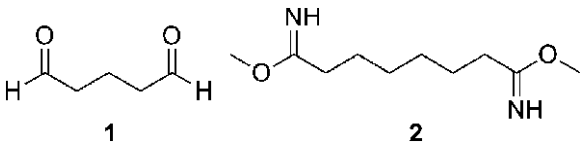

Fig. 2 Chemical structure of glutaraldehyde (**1**) and dimethyl suberimidate (**2**) utilized for amine–amine crosslinking by Torchilin et al. [124, 125]

expected. This may interfere with the binding of the antibody to its receptor and thus alter the binding affinity.

2.2 Coupling of Ligands to Carboxylic Acid-Modified Liposomes

Covalent coupling of ligands to carboxylic acid-modified liposomes (Fig. 1b) is a widely used approach to functionalize liposomes [14, 16, 63, 74, 86, 99, 143]. The method was first introduced by Kung and Redemann [74], who introduced the carboxylic acid functionality by reacting PE-lipids with a wide range of anhydrides in presence of triethylamine. Liposomes, exposing the carboxylic acid functionality, were activated in situ by the addition of water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The activated ester was found to react readily with primary amines present on mouse IgG resulting in a stable amide bond. The achieved coupling efficiencies ranged from 1 to 58%, depending on the length of the spacer between the liposome surface and the carboxylic acid functionality.

The effect of the spacer becomes negligible when the ligand is introduced at the PEG terminus, as described by Maruyama et al. [86], who prepared monoclonal IgG 273-34A-modified liposomes by the above-described approach. These immunoliposomes were found to have prolonged circulation times and a higher degree of target cell recognition compared to liposomes exposing antibodies directly on the liposome surface.

The major advantage of using carboxylic acid-modified liposomes for surface modification purposes is that no prior ligand modification is required, thus reducing the risk of denaturation. On the other hand, since multiple amine functionalities are present in antibodies, a random attachment can be expected, which could alter the binding affinity towards the targeted receptor.

2.3 Coupling of Ligands to Aldehyde-Modified Liposomes

Coupling of hydrazino-derivatized ligands to aldehyde functionalized liposomes by hydrazone formation (Fig. 1c) has been described by Bonnet et al. [20], who

introduced the aldehyde functionalized ether lipid di-*O*-hexadecyl-*rac*-glyceraldehyde into the liposomal membrane. Incubation with a hydrazino-derivatized dodecapeptide from the cytoplasmic domain of lysosome-associated membrane protein (LAMP) in aqueous buffer resulted in quantitative conversion within 5 h.

The method described by Bonnet et al. [20] offers an effective approach to conjugate synthetic peptides prepared by solid-phase peptide synthesis (SPPS) to liposomes. The hydrazino functionality is easily introduced to the synthetic peptide on resin by the use of *N*,*N*,*N*-tri(*tert*-butyloxycarbonyl)-hydrazino acetic acid, which is fully compatible with SPPS synthesis [18, 19]. Furthermore, hydrazone formation occurs spontaneously without the need of a catalyst. Thus, this method is one of the most effective for the functionalization of liposomes with targeting ligands when it is possible to introduce a hydrazino group into the ligand. However, this is unfortunately problematic for antibodies and other complex ligands.

2.4 Coupling of Ligands to Hydrazide-Modified Liposomes

A more widely adapted approach, compared to the one described above, is to invert the position of the functional groups, i.e., introducing the aldehyde to the ligand and the hydrazine functionality to the liposome surface (Fig. 1d) [26, 56, 57, 72, 91, 145, 146]. Initially, the hydrazine functionality was introduced by incorporation of lauric acid hydrazide [26] into the liposome membrane (it should be noted that this is a poor anchor). Later, a method to introduce the hydrazine functionality to the distal end of the PEG chain of DSPE–PEG has been described by Zalipsky [145]. Having the hydrazine functionality exposed on the liposomal membranes offers a unique advantage for coupling of antibodies to the liposomal membrane. Mild oxidation of the carbohydrate groups on the constant region of the heavy chain of the immuoglobulin with either galactose oxidase [26] or sodium periodate [26, 56, 57, 72, 91] results in the formation of an aldehyde, which can be chemoselectively attached to the hydrazine-functionalized liposomes through hydrazone bond formation. By utilizing the carbohydrate groups from the Fc region, the antibodies are correctly oriented once attached onto the surface of the liposomes, because only the Fc region is involved in the coupling reaction, leaving the antigen-binding site available for receptor interactions. Comparative studies have indicated that this method results in low coupling efficiencies (17%) [56], yet positive in vitro results have been obtained using this methodology with liposomes targeted towards rat colon carcinoma CC531 cells [72].

2.5 Coupling of Ligands to Maleimide-Modified Liposomes

The most often used approach to functionalize liposomes with targeting ligands is based on the formation of a thioether bond between maleimide-functionalized liposomes and thiol-derivatized ligands [13, 29, 34, 40, 42, 49, 64, 70, 85, 98, 101,

119] by Michael Addition (Fig. 1e). For direct surface functionalization, *N*-(4-(*p*-Maleimidophenyl)butyryl)-phosphatidylethanolamine has been used as the functionalized anchor, whereas DSPE–PEG–maleimide is used for attachment on the distal end of the PEG polymer. Garnier et al. [49] recently used this approach to covalently attach the Annexin-A5 protein, known to target membranes containing negatively charged phospholipids, to DSPE–PEG–maleimide functionalized liposomes. A mutant of the natural Annexin-A5 protein (35 kDa), exposing a cysteine residue at a highly accessible loop on the concave face of the protein, was developed. Addition of this protein to liposomes exposing the maleimide functionality in HBS buffer at pH 6.3 for 4 h resulted in a coupling efficiency of approximately 80%. The Annexin-A5-functionalized liposomes were found to bind to solid supported lipid membranes composed of DOPC/DOPS in a Ca^{2+} depending manner, as monitored by quartz crystal microbalance (QCM). Michael addition of thiolated OX26 MAb Fab′ fragments to DSPE–PEG–maleimide functionalized liposomes has recently been described by Béduneau et al. [13]. Despite optimizing the coupling conditions, the coupling yield was constantly approximately 25%. However, it is noteworthy to mention that quantitative coupling efficiencies have been reported with small thiolated pentameric cRGD peptides using a similar coupling protocol [64].

An interesting study performed by Fleiner et al. [40] concerning the influence of the spacer length between the liposome surface and the reactive maleimido group and its polarity revealed that longer polar spacers resulted in higher coupling efficiencies. Surprisingly, comparison of the reactivity of liposome functionalized with either *m*- or *p*-maleimido benzoic acid esters revealed that the less reactive (less electrophilic) *m*-maleimido benzoic acid ester resulted in a higher coupling efficiency (46 ± 7%) compared to the more electrophilic *p*-maleimido benzoic acid analogue (30 ± 5%). This could be explained by the increasing susceptibility to competing nucleophiles, such as water, of the maleimide group with higher electrophilicity.

Surface conjugation to maleimide-functionalized liposomes is a straightforward and reliable method to attach ligands without prior activation or addition of catalysts to promote the reaction. The reaction proceeds at ambient temperature, close to neutral pH and within a short period of time. Conjugation of smaller ligands often results in quantitative yields, whereas more moderate yields can be expected for larger molecules. Despite the popularity of this conjugation method, maleimide derivatives have been shown to be immunogenic [17, 106].

2.6 Coupling of Ligands to Thiol-Modified Liposomes

Thiol-functionalized liposomes have often been used for the attachment of ligands to the outer liposomal membrane. Normally, the reactive thiol is introduced to the membrane as the disulfide protected derivate *N*-(3′-(pyridyldithio)propionoylamino–PEG–DSPE (DSPE–PEG–PDP), which is activated in situ by reduction with

dithiothreitol (DTT), as described by Allen et al. [5]. Attachment of maleimide-derivatized antibodies (Fig. 1f) was achieved by overnight incubation in yields ranging from 13 to 88%, depending on the liposome composition and amount of maleimide-derivatized antibodies added. Quantitative conjugation yields with maleimide-derivatized My10 antibodies have been reported when the reactive thiol is introduced at the distal end of longer PEG chains than the ones otherwise present in the liposome [92].

Surface conjugation to thiol-modified liposomes can also be achieved by disulfide formation (Fig. 1g). This approach was adopted by Muñoz et al. [96], who introduced the hepatitis A VP3 (101–121) peptide to DSPE–PEG–PDP containing liposomes. Overnight incubation in borate buffer at pH 8 resulted in approximately 50% conjugation yield. A disadvantage of this approach is that free thiols may react among themselves to produce intermolecular disulfide bonds, leading to crosslinking of the reactive ligands or liposomes.

2.7 Coupling of Ligands to Bromoacetyl-Modified Liposomes

Conjugation of cysteine-containing peptides to bromoacetyl-modified liposomes (Fig. 1h) has been described by Frisch et al. [42]. The bromoacetyl functionality was introduced by acylation of DPPE with 2-[2-[2-[(2-bromoacetyl)amino]ethoxy]ethoxy]ethoxy acetic acid (**3**) (Fig. 3) in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC). An octapeptide derivatized from the C-terminal of the histone H_3 peptide was added to liposomes, exposing the bromoacetyl functionality at pH 9.0 resulting in quantitative conversion within 1 h. At lower pH, the reaction was found to be less pronounced. This phenomenon was utilized by Schelté et al. [119], who formulated liposomes exposing both the maleimide- and the bromoacetyl functionality at the outer membrane. This study showed that important kinetic discrimination can be achieved between the maleimide and bromoacetyl functionalities when the reactions with thiols are performed at pH 6.5. Reaction with cysteine-containing peptides was found to be three orders of magnitude faster with the maleimide functionality than with the bromoacetyl derivative, resulting in a high degree of chemoselectivity. These findings enabled the coupling of two different cysteine peptides sequentially. The first coupling was carried out on the maleimide derivative at pH 6.5, followed by a coupling to the bromoacetyl derivative at pH 9.0 under experimental conditions, which were found

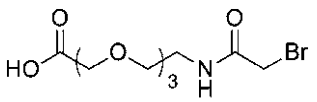

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Fig. 3 Chemical structure of 2-[2-[2-[(2-bromoacetyl)amino]ethoxy]ethoxy]ethoxy acetic acid (**3**) used to introduce the acetyl bromo functionality to DPPE, as described by Frisch et al. [42]

not to alter the integrity of the liposomes. Furthermore, neither the bromoacetyl nor the maleimide functionality was found to react with other nucleophiles such as α- and ε-amino groups or imidazole, which could also be present in peptides.

2.8 Coupling of Ligands to Cysteine-Modified Liposomes

Conjugation of recombinant proteins to the liposome surfaces through native chemical ligation (Fig. 1i) has recently been described by Reulen et al. [109]. Native chemical ligation was first reported by Dawson et al. [32] as a unique method to ligate two unprotected peptide fragments and form an amide bond, thereby facilitating the synthesis of large proteins. Native chemical ligation is a chemoselective reaction, which occurs spontaneously between a thioester and an N-terminal cysteine under aqueous conditions at neutral pH, which makes it ideally suited for liposome conjugation purposes. Reulen et al. [109] introduced the cysteine functionality to the distal end of DSPE–PEG–NH_2 by reacting it with succinimidyl-activated trityl-protected cysteine, followed by deprotection of the trityl protection groups with dilute trifluoroacetic acid (TFA). The C-terminal of the collagen-binding protein domain (CNA35) from the bacterial adhesion protein of *Staphylococcus aureus* was modified with sodium 2-mercaptoethanesulfonate (**4**) (MESNA) (Fig. 4) to form a thioester suitable for native chemical ligation. Cysteine-functionalized liposomes were incubated with MESNA-CNA35 protein in HBS buffer at pH 8 for 48 h in the presence of thiophenol, benzyl mercaptan or MESNA to catalyze the reaction. In the presence of either thiophenol or benzyl mercaptan, approximately 30% conversion was observed, whereas only 10% conversion was achieved with MESNA. However, the poorly water soluble and toxic thiophenol and benzyl mercaptan were found to accumulate in the phospholipid bilayer, making them difficult to remove after ligation. This was not the case with the water-soluble MESNA, which was easily removed by centrifugation. The CNA35-functionalized liposomes were tested in a collagen-binding assay and were found to have a 150-fold increase in affinity compared to the free protein.

Surface conjugation through native chemical ligation is an attractive method to directly conjugate thioester-modified ligands to the liposome surface. The method enables site-specific conjugation, since only a single site in the ligand is available for conjugation. The low coupling yield is an obstacle that needs to be addressed.

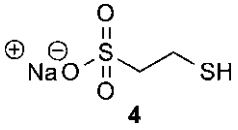

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Fig. 4 Chemical structure of sodium 2-mercaptoethanesulfonate (**4**) (MESNA) used to catalyze the native chemical ligation on the liposome surface, as described by Reulen et al. [109]

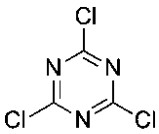

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Fig. 5 Chemical structure of cyanur chloride (**5**) used to introduce the cyanur functionality into liposomes by acylation of DPPE–PEG–OH, as described by Brendas et al. [21]

2.9 Coupling of Ligands to Cyanur-Modified Liposomes

A method for attaching antibodies directly to the PEG terminus of liposomes without prior derivatization has been described by Brendas et al. [21]. Introduction of the cyanur functionality to the PEG terminus of DPPE–PEG–OH was achieved under basic conditions by adding cyanuric chloride (**5**) (Fig. 5). Anti E-selectin monoclonal antibodies were coupled to cyanuric-modified liposomes (Fig. 1j) at pH 8.8 by nucleophilic substitution resulting in immunoliposomes having a high degree of in vitro binding to Chinese hamster ovary cells expressing E-selectins receptors. The nucleophilic substitution between the anti-E-selectin monoclonal antibodies and the cyanur-modified liposomes was found to be very sensitive towards the pH of the buffer. No surface functionalization was observed at neutral pH, whereas a more alkaline environment resulted in hydrolytic degradation of the cyanuric chloride.

This methodology offers a straightforward approach for attaching antibodies to the PEG terminus of liposomes without previous derivatization. However, cyanuric chloride is known to react with a wide range of nucleophilic functionalities, such as alcohols, amines and thiols, which means that a random attachment of the antibodies can be expected. This may interfere with the binding of the antibody to its receptor, thus altering the binding affinity. In addition to this, cyanuric chloride is regarded as a sensory respiratory irritant [113], but has not shown any sign of acute, chronic or genotoxicity [140].

2.10 Coupling of Ligands to p*-Nitrophenylcarbonyl-Modified Liposomes*

An additional method to directly conjugate antibodies to the PEG terminus of liposomes without prior derivatization has been described by Torchilin et al. [128]. The amphiphilic derivate *p*-nitrophenylcarbonyl–PEG–1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (*p*NP–PEG–DOPE), which was obtained in a single step from DOPE and bis(*p*-nitrophenylcarbonyl)-PEG, forms stable and non-toxic carbamate bonds with ligands containing primary amines (Fig. 1k). However, one drawback is the utilization of the bis-functionalized bis(*p*-nitrophenylcarbonyl)-PEG, which may result in dimerization, although this side product is easily

separated from the product. Torchilin et al. demonstrated this approach with several proteins, such as concanavalin A (ConA), wheat germ agglutinin (WGA), avidin, monoclonal antimyosin antibody 2G4 (mon2G4) and monoclonal antinucleosome antibody 2C5 (mon2C5), and observed almost quantitative surface functionalization yields at pH 8.0. Despite the fact that this method does not enable site-specific conjugation, the specific activities of the surface bound proteins were retained after conjugation.

2.11 Coupling of Ligands to Alkyne-Modified Liposomes

One of the more elegant coupling methods to functionalize liposomes is based on the work of Meldal and co-workers [129, 130] and Sharpless and co-workers [111], who reported the use of Cu(I) to catalyze the azide/alkyne Huisgen 1,3-dipolar cycloaddition, commonly referred to as the click reaction. This reaction offers unique flexibility because of the high level of orthogonality to other chemical functionalities and generally proceeds rapidly in high yields. This approach to functionalize liposomes was first described by Hassane et al. [58], who introduced the alkyne functionality to the liposome surface by incorporating the synthetic ether lipid *N*-[2-(2-(2-(2-(2,3-bis(hexadecyloxy)propoxy)ethoxy)ethoxy)-ethoxy)ethyl]hex-5-ynamide (**6**) (Fig. 6) into the liposomal membrane (Fig. 1l). Addition of an azido-modified mannose ligand in the presence of $CuSO_4$ and sodium ascorbate to generate Cu(I) in situ resulted in approximately 25% yield within 24 h. However, by adding the water-soluble Cu(I)-stabilizing ligand bathophenanthroline disulfonic acid [78] to the reaction mixture, complete conversion was observed within 6 h. These reaction conditions did not alter the size of the liposomes or provoke leakage from liposomes loaded with self-quenching concentrations of 5,6-carboxyfluoroscein. Furthermore, the mannose residue was found to be readily accessible to concanavalin A, which upon addition to the liposomes caused instant aggregation.

The click reaction approach has also been adopted by Cavalli et al. [23], who introduced the alkyne functionality by derivatization of DOPE with proliolic acid. Full conversion was in this case achieved within 20 h with a small azido-NBD derivative without the use of bathophenanthroline disulfonic acid.

The Cu(I) catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition is a very powerful conjugation reaction for surface modification of liposomes. The

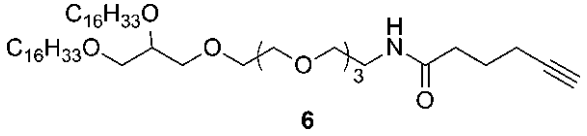

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Fig. 6 Chemical structure of *N*-[2-(2-(2-(2-(2,3-bis(hexadecyloxy)propoxy)ethoxy)ethoxy)-ethoxy)ethyl]hex-5-ynamide (**6**) used to introduce the alkyne functionality into the liposome membrane as described by Hassane et al. [58]

unreactive nature of both alkynes and azides towards the other functionalities present in biomolecules makes site-specific conjugation possible. Furthermore, the formed triazole ring is both thermal and hydrolytically stable, and the reaction can occur efficiently in aqueous media at room temperature. However, the mandatory use of copper catalyst represents a limitation. Copper is considered toxic and complete removal prior to in vitro or in vivo use is necessary. In addition to this, unsaturated phospholipids are known to be oxidized by copper ions in the presence of oxygen [46, 75], which could cause degradation of the liposomal membrane. Alternative methods to promote triazole formation between azides and alkynes, without Cu(I), have been described recently. Strain-promoted [3] or electron-deficient alkynes [79] have been reported to react with azides in absence of Cu(I), but these methods have not yet been applied to the liposome field.

2.12 Coupling of Ligands to Triphosphine-Modified Liposomes

The latest member of the wide range of possible surface conjugation reactions described is based on the Staudinger ligation [118], in which an azide and a triphosphine selectively react to form an amide bond (Fig. 1m). This approach was adopted by Zhang et al. [148], who introduced the triphosphine functionality by acylation of DPPE with 3-diphenylphosphino-4-methoxycarbonylbenzoic acid (**7**) (Fig. 7). Triphosphine-functionalized liposomes were incubated with an unprotected lactosyl derivate carrying an ethyl spacer functionalized with an azide group in PBS buffer, which resulted in 80% surface functionalization within 6 h. The surface conjugation reaction was not found to alter the size or provoke leakage of the liposomes. The surface-conjugated lactose residues were shown to be easily accessible to β-galactose-binding lectin, which, upon addition, caused aggregation of the liposomes.

The methodology described by Zhang et al. [148] offers an efficient and chemoselective conjugation method for liposome surface functionalization. The reaction benefits from being performed under mild conditions without the need of a catalyst. Furthermore, methods to engineer bacteria and yeast enabling them to incorporate azido functionalities into proteins have been developed [53]. This enables direct attachment to the triphosphine-functionalized liposome without prior derivatization of the protein.

3 Targeting Strategies: Active Targeting of Tumor Vasculature and Tumor Cells

Ligand-modified liposomes can be designed to target receptors expressed by cells in the tumor vasculature or on the tumor cells. When targeting tumor cells directly,

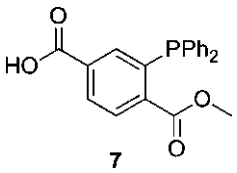

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Fig. 7 Chemical structure of 3-diphenylphosphino-4-methoxycarbonylbenzoic acid (**7**) used to introduce the triphosphine functionality into liposomes by acylation of DPPE as described by Zhang et al. [148]

a number of obstacles have to be overcome as the drug delivery system has to cross the vasculature and travel through the interstitium in the tumor tissue before being able to deliver the drug at the desired site of action. Vascular targeting is generally considered to be advantageous over tumor cell targeting as endothelial cells are genetically stable and the risk of developing drug resistance is minimized [1]. Proliferating endothelial cells in solid tumors share similar characteristics in a variety of tumor types and a number of these can be utilized. In addition, endothelial cells lining the blood vessels in tumors are more accessible for binding by circulating drug delivery systems that are administered intravenously [1]. A strategy that targets both the tumor vasculature and the tumor cells has been envisioned as more effective than targeting the two tissues individually, and Koning et al. have provided proof-of-principle for this hypothesis [73]. These strategies have been investigated using a range of targeting molecules including antibodies, peptides, carbohydrates and vitamin analogs, and an overview of these different targeting moieties is discussed below.

3.1 Immunoliposomes

Antibodies represent one of the most versatile ligands that can be attached to liposomes. However, the antibody functionalization of liposomes is generally challenging, where region specificity and degree of conversion are the main problems, even though a number of good methods exist. One of the main reasons for this is that antibodies can only be attached to liposomes by *post-functionalization*, i.e., the lipsomes are formed prior to attachment, and the chemistry therefore has to work well in aqueous buffer. Early attempts to attach these molecules for the specific targeting of diseased tissue were originally communicated in the 1970s [136] where antibodies were coupled to the surface of non-PEGylated liposomes. Today, it is commonly known that non-PEGylated (or non-polymer) liposomes have little treatment benefit because of fast clearance from the blood stream. Coupling of the targeting ligands to the distal end of PEG is therefore the method-of-choice today to maximize liposome accumulation at the target site [56, 116]. Antibodies can be attached as whole monoclonal antibodies or as a fragment thereof (Fig. 8). Full antibodies have stability advantages over small

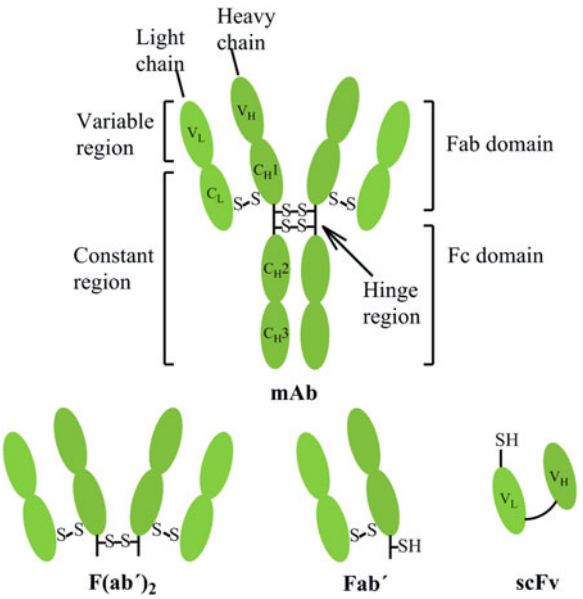

Fig. 8 Schematic representation of various antibody constructs: Monoclonal antibody (mAb); $F(ab')_2$ generated by pepsin digestion of the Fc domain of mAb; Fab′ from reduction of the disulfide bond in the hinge region of $F(ab')_2$; scFv of recombinant V_L and V_H regions linked by a short peptide sequence

fragments such as Fab′ and scFv, but can trigger complement [94] and induce antibody-dependent cell-mediated cytotoxicity [115]. Furthermore, the Fc fragment is known to accelerate clearance of the immunoliposome by uptake of circulating liver and spleen macrophages possibly through opsonization, and thus decreases the circulation time of the liposomes [5]. By attaching fragments of the antibody such as Fab′ or Fv, this undesired clearance can be minimized [12].

The most commonly used antibody-targeting moieties belong to the immunoglobulins of the IgG class [126], which have been coupled to the distal end of PEG using a hydrazido-PEG–DSPE construct [57]. The oligosaccharide moiety of the antibody molecule was oxidized using sodium periodate, creating an aldehyde functionality that reacts with the hydrazido-group on the PEG–DSPE to form a stable hydrazone linkage. Immunoglobulins consist of 82–96% polypeptide and 4–18% carbohydrates [10, 11], and the carbohydrate moieties are mainly situated on the heavy chain in the Fc portion of the antibody. As this region is not involved in the binding to the receptor, a modification in this part should not influence antigen-binding efficacy [52]. However, one potential problem with the oxidation of the carbohydrates is the risk of oxidizing amino acids situated in the antigen-binding Fab′ region. Amino acids most prone to oxidation with sodium periodate are cysteine, methionine, tryptophan, tyrosine and histidine, as well as serine and threonine if they occur as terminal residues [28].

Antibodies against the human epidermal growth factor receptor 2 (HER2) have been widely used in combination with liposomal drug delivery systems. This antigen is frequently overexpressed on various types of cancer cells and is only weakly expressed in normal tissues. One of the first studies of investigating Fab′

and scFv targeting of HER2 was reported by [85]. They utilized PEGylated liposomes that were functionalized with a maleimide functionality in the distal end of PEG. Fab′ was conjugated to the liposomes through a thioether linkage using the free thiol group in the Fab′ hinge region at pH above 7 to deprotonate the thiol. This site is located distant from the antigen-binding site and should not interfere with its function. The thioether bond formed between Fab′ and the maleimide functionalized liposome was stable and not prone to reduction in a reductive environment, e.g., serum [84]. ScFv fragments contain immunoglobulin heavy- and light-chain variables linked by a $[\text{-}(\text{Gly})_4\text{-Ser-}]_3$ motif. At the C-terminal end of the recombinantly produced scFv, a cysteine can be introduced that can be exploited in the covalent attachment to maleimide-activated PEG–DSPE. Two internal disulfide bonds are present in the scFv fragment; however, these have proven to be rather stable to reduction [4]. An analogous approach has been developed with the use of a PDP–PEG–DSPE liposome composition. After reduction with DTT, the liposome was incubated with an anti-HER2 maleimidophenylbutyrate functionalized Fab′ fragment, resulting in the immunoliposome [47]. For targeting the transferrin receptor (TfR), its antibodies or Fab′ fragments have been coupled to maleimide-modified PEG–DSPE liposomes. The TfR expression is elevated in many types of cancerous tissues and correlates with the proliferation rate and aggressiveness of the cancer. Hence, the TfR is a valuable potential target for drug delivery in cancer therapy [13, 141].

For the targeting of endothelial cells in malignant tissues, different forms of adhesion molecules such as endothelial-leukocyte adhesion molecule-1 (ELAM), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) have been applied. These have been coupled to DOPE-*N*-dodecanoyl lipids by the use of EDC/*N*-hydroxysuccinimide (NHS) chemistry [55]. However, as there are several amine groups in anti-ELAM and anti-ICAM, this strategy leads to a non-specific crosslinking between the liposome and the antibody. This can be avoided by applying the maleimide method described above where the antibody is coupled via a thioether bond to the surface of PEGylated liposomes. However, an additional step is required to introduce a thiol functionality on the cell adhesion molecule. This is readily done using *N*-succinimidyl-*S*-acetylthioacetate [35]. Related to this strategy, immunoliposomes have been prepared using maleimide–DPPE through a reaction with thiolated $F(ab')_2$ fragments of the monoclonal antibody GAH [131]. GAH recognizes surface molecules especially in stomach cancer, and a PEGylated immunoliposome formulation using GAH targeting has been shown to have better efficacy than non-targeting liposomes in Phase I clinical trials [59, 88].

A significant number of monoclonal antibodies have been identified and can be engineered as Fab′ or scFv fragments as well as chimeric or humanized antibodies. Highly effective and reproducible methods of coupling whole antibodies or their fragments to the surface of plain liposomes or PEGylated liposomes exist, and in general immunoliposomes show an enhanced recognition of target cells, as well as a better internalization and intracellular drug delivery over non-targeted liposomes [71, 102].

3.2 Folate-Modified Liposomes

Liposomes functionalized with the vitamin, folic acid, have attracted much attention as the folate receptor is highly overexpressed in a number of cancers, and it can retain its high affinity binding to the folate receptors even after being covalently linked to a variety of macromolecules [144]. The vitamin is internalized to a large extent in proliferating cells as folic acid is essential in the biosynthesis of nucleotides; consequently, cancerous cells overexpress the folate receptor as the fast-dividing malignant cells are in great need of this nutrient. Normally, the folate receptor is expressed at the apical side of epithelial cells and is not accessible to blood-borne drugs. However, when the epithelial cells become malignant, the membrane loses its polarity and the folate receptor can be located at the basal surface of the membrane as well, taking up therapeutics from the plasma [81]. There is no evidence that normal healthy cells are expressing the folate receptor to a significant extent, and healthy tissue should therefore not be affected by the delivery of folate-targeted drugs [103, 138]. The first report of using folate-targeted liposome to transport drugs into tumor cells was published in the 1990s by Lee and Low [76]. They conjugated the NHS-ester of folic acid to the distal end of a NH_2-PEGylated liposomes forming a stable amide bond. Gabizon et al. applied a similar approach simply using a DCC-mediated coupling procedure linking the terminal free γ-carboxylic acid on folate and the free PEG amine functionality [44]. Folate contains both an α- and γ-carboxylic acid moiety, but as the gamma position has a higher reactivity, this is the main coupling product observed [68]. After endocytotic uptake [9] of the liposome complex by the folate receptor-expressing cell, release of the liposome content has been accomplished taking advantage of the endosomal acidic environment, either by introducing pH-sensitive fusogenic peptides to promote uptake into the endosome [134] or adding pH-sensitive lipids to the liposome to increase liposome permeability and accelerate unloading of the content in the acidic endosome [112]. The folate molecule can form dimers, trimers and even higher self-assembling constructs. An increasing level of folate attached to the liposome surface was found not to increase the level of folate–liposome-complex binding to the folate receptor, as this receptor can only bind one folate and will not respond to multiple folates [27, 107]. One of the advantages with folate targeting is that the lipid–PEG–folate conjugates can be synthesized in organic solvent and purified. This allows for a precise control of the amount of targeting ligands that are present in the liposome, and there is no need for *post-functionalization*.

3.3 Saccharides

A large number of mammalian cells express sugar-binding proteins known as lectins. These have been found to be overexpressed on malignant cells and are believed to be involved in metastasis formation [31, 43]; they can thus serve as a

target for drug delivery systems. Liposomes functionalized with carbohydrates are biodegradable, low in toxicity and have a protein-shielding ability that makes them able to minimize liposome clearance from circulation. For instance, monosialoganglioside have been shown to enhance the circulation time similar to that observed for PEGylated liposomes [8, 90]. A number of different carbohydrates have been conjugated to the surface of liposomes. Hassane et al. [58] used click chemistry to couple an unprotected α-D-mannosyl derivative carrying a PEG spacer functionalized terminally with a reactive azide moiety. However, selective protection of the mannose alcohol groups is naturally required during functionalization with the PEG chain to avoid multiple substitutions [58]. Surface functionalization of liposomes using lactose, galactose and a diverse array of polysaccharides as the carbohydrate components have been synthesized using Staudinger ligation [148], thioglycoside-mediated attachment [147] and EDC couplings between the headgroup amine on the lipid, and an activated NHS-ester of the saccharide moiety [123, 142]. The employed methods have both involved *post-functionalization* and complete synthesis of the targeting lipid–ligand conjugate in organic solvent, allowing isolation of the target compound. The latter method is possible with all carbohydrate targeting ligands and should be the method-of-choice.

3.4 Peptides

The use of peptide-targeted liposomes as therapeutics has become highly interesting with the increasing knowledge of specific peptide sequences of proteins involved in cell–cell interactions as well as the improvements in synthesis or expression of synthetic peptides that closely resemble the human ones. For attachment of peptide moieties to the liposome surface, the typically reactive amino acids used are lysine, serine, cysteine, histidine, arginine, aspartic acid, glutamic acid, threonine, tyrosine, the N-terminal amino group and C-terminal carboxylic acid. The primarily used method is to react one of these reactive functionalities with an activated PEG component situated on the surface of the liposome. As lysine is one of the most prevalent amino acids in proteins, this has been a favored linking site; however, upon reaction of a nucleophilic amine group with an electrophilic activated PEG, multiple substitutions are often observed due to the the presence of multiple lysines in the peptide. Amine linkage to PEG has been achieved in multiple ways including the use of PEG dichlorotriazine [2] and PEG tresylate [41]. Many PEG conjugates have furthermore been synthesized using activated carbonate derivatives, such as succinimidyl carbonate, trichlorophenyl carbonate and *p*-nitrophenyl carbonate [93, 132]. Liposomes have been modified with cell-penetrating peptides such as TAT using a *p*-nitrophenyl carbonate-functionalized PEG under slightly basic conditions to form the non-toxic carbamate linkage. TAT and other cell-penetrating peptides such as penetratin and synthetic polyarginines have been conjugated to the surfaces of liposomes to improve liposome uptake by cells [127]. In general, lower reactivity of the

carbonate reagents provides higher selectivity; however, carbonate linkage with several amino acids such as lysine, histidine and tyrosine have been observed [110]. When a highly selective conjugation is required, a PEG–propionaldehyde can be prepared. This functionality will, if pH is controlled, react selectively with the N-terminal α-amine because of the lower p*K*a value of this amine compared to other possible nucleophiles in the sequence [69]. Another possibility is to make the activated ester of PEG carboxylic acids, such as NHS–PEG, which will react with primary amines to form stable amides. This method has been applied for the modification of liposomes with the vasoactive intestinal peptide (VIP) to target VIP receptors in breast cancer [30]. The receptor for this 28-amino acid neuropeptide is found to be overexpressed and homogenously distributed in all parts of the breast tumor and thus serves as a potential tumor-targeting functionality [108]. Other active targeting carriers are based on delivery systems that mimic local bioadhesion. Integrins and in particular the $\alpha_5\beta_1$ integrin have attracted much attention as this and its ligand, fibronectin, are found to be upregulated in blood vessels in tumor biopsies. There is strong evidence that peptides that mimic the cell adhesion domain of fibronectin and contain the peptide sequence RGD (arginine-glycine-aspartic acid) are potent inhibitors of tumor-growth, tumor metastasis and tumor-induced angiogenesis [89]. The RGD sequence has been prepared as a cyclic constrained 5-mer modified with a C-terminal thioacetyl group for linkage to the liposome. Deprotection of the acetyl group and incubation with a maleimide-PEGylated functionalized liposome resulted in a thioester linkage between the peptide and liposome [120]. A similar approach was used for the attachment of the linear ATWLPPR (alanine-threonine-tryptophan-leucine-proline-proline-arginine) sequence, found to have affinity for the vascular endothelial growth factor receptor (VEGF), a receptor overexpressed in the surface of angiogenic endothelial cells [15]. Utilization of cysteine residues is the main approach for site-specific modification due to high specificity and ease of modification of a sequence that lacks a cysteine residue. In addition, few free cysteines are present on the surface of proteins compared to, e.g., lysine. An additional synthetic peptide sequence often applied in blocking cancer cell adhesion is YIGSR (tyrosine-isoleucine-glycine-serine-arginine)—a sequence shown to be important in laminin receptor binding. Conjugation of the peptide moiety to the liposomal surface can be achieved by mild periodate oxidation of the threonine residue in the hexapeptide TYIGSR, leading to a reactive glyoxylyl functionality in the N-terminal end of the peptide followed by attachment to a hydrazide functionalized PEG lipid [146]. The oxidation of carbohydrate residues or N-terminal serine or threonine creates the possibility for site-directed PEGylation using hydrazides. The glyoxylyl functionality formed by the oxidation of N-terminal serine or threonine reacts site-specifically with PEG-hydrazide derivatives [45], and the PEGylated peptide can be purified before use. A similar site-specific N-terminal modification of peptides has been described by Geoghegan and Stroh [50]. In general, the peptides discussed above can be achieved in several ways and can both be inserted by *post-functionalization* and by synthetic procedures in organic solvent. The former is an advantage for larger peptides (>30

amino acids) that are relatively expensive as the purification step utilized in the latter procedure results in loss of compound. However, a synthetic approach that involves purification should always be employed when possible to remove side products and reagents. A number of reports furthermore utilize SPPS of lipopeptide conjugates, and this is a highly efficient method for smaller peptides [66].

4 Membrane Anchors

In relation to liposomes and other self-organized materials, an important consideration when choosing the chemical structure of the targeting conjugate is that membrane affinity for the specific lipid membrane has to be sufficiently high to secure stability during blood circulation and binding to the target receptor. The easiest strategy is of course to choose the same lipid anchor for the targeting conjugate as the majority of the lipids used in the formulation; however, this may not always be possible, and for large hydrophilic targeting conjugates it may not always be sufficient. Depending on the targeting conjugate and the concentration used, the packing parameter of the molecule should also be considered [83]. The flexibility of the functional group as well as the crowding from other molecules on the membrane surface will influence the conformation of the anchored group, e.g., brush-like versus mushroom-like structures of PEG [48]. Two of the most important factors in choosing an anchoring molecule are the critical aggregation concentration (CAC) of the targeting conjugate and how compatible it is with the lipid membrane in terms of membrane thickness and fluidity, e.g., saturated versus unsaturated hydrocarbon chains. It should be noted that using poly-unsaturated hydrocarbon chains might induce a stability challenge, since these are prone to oxidation [133]. A simple example of the importance of fluidity is that rhodamine-labeled DOPE in a DSPC liposome membrane migrates in cell culture to the cell membrane, whereas rhodamine-labeled DSPC remains bound to the liposomes. It is therefore highly important to evaluate the physico-chemical properties of the targeting conjugate and measure the formulation stability with appropriate biophysical methods.

A large variety of lipids has been used to anchor different targeting ligand conjugates in liposomal membranes. Among the most widely used are phospholipids (PL) [137], cholesterol [22], ether lipids [38], acyl chains [60], glycophosphatidylinositol (GPI) [121] and molecular rods [51]. Each anchor type has several subtypes where small modifications can have a significant influence on how well the anchor of the functionalized group associates with the membrane. The choice of anchor depends on the physico-chemical properties of the conjugated group.

Phospholipids are the most widely used anchors since most liposome-based drug delivery systems are composed of commercially available PLs. PE and PE–PEG–NH_2 are the most utilized when synthesizing targeting moieties by

standard chemistry in organic solvent. The linkage chemistry of choice is often activated carbonates, and "click" chemistry is utilized more and more often due to the high level of functional group orthogonality. However, hydrazines should also be considered for reaction with aldehydes as this chemistry works very well and is highly orthogonal to other chemistries. For *post-functionalization*, Michael addition to maleimides with thiols is by far the most often used approach as this chemistry is fully compatible with the aqueous environment. "Click" chemistry can also be considered, but it is more difficult to achieve high coupling efficiency between sterically hindered moieties in comparison to the maleimide chemistries. A special case of PLs is the sphingolipids, which have three major subclasses: (1) ceramides, (2) sphingomyelins and (3) glycosphingolipids, where the latter is mainly used to anchor large proteins. One drawback of using glycerophospholipids as anchors is that enzymes that hydrolyze the lipids giving free fatty acid and lysophospholipids can be overexpressed in diseased tissue, e.g., phospholipase A2 (PLA2) [7, 65]. The enzyme hydrolysis will eventually disrupt the lipid membrane, and the liposome thereby loses its targeting capacity. To circumvent problems with enzymatic hydrolysis or chemical degradation of the ester bonds (which are the weakest chemical bond in the usual phospholipid conjugates), ether lipids can be used. The chemistry of conjugation is the same as for the normal diacyl-glycerophospholipids, but the cost of manufacturing represents a limitation.

Cholesterol and derivatives like thio-cholesterol are also among the frequently used anchors for functional groups on the surface of liposomes. Like PLs, cholesterols are widely used in liposomal drug delivery systems and are also highly present in natural membranes, making cholesterol anchors highly compatible with most membranes. Cholesterol is typically coupled to a functional group via an ester bond, carbamate ester or ether bond using the same type of chemistry as for PLs. Cholesterol will usually serve as a strong anchoring molecule to relatively large targeting conjugates with a low CAC value.

Acyl chains are also commonly used as anchors due to the easy coupling to primary amines and alcohols. The stability of the amide bond makes this approach suitable for solid phase synthesis and usually involves only one acyl chain, but in some cases it involves two or three [80].

The use of only one acyl chain has been seen in multiple studies, but does not generally serve as a sufficient anchor in vivo (or even in vitro) as the water solubility is too high and fast migration to, e.g., cell membranes will be observed. If mono-acylation is used, particular attention should always be given to verifying the anchoring stability.

Molecular rods are a relatively new form of anchors for lipid membranes with functional groups attached to each end. They are designed to fit into membranes spanning the entire bilayer, unlike most other lipid anchors, and can be synthesized with various lengths and desired rigidity needed for the individual target membrane [97]. The principle of lipids spanning the entire membrane is known from archaebacterial membranes, and mimics of the natural occurring lipids have been synthesized [104]. Both molecular rods and tetraether lipids have the advantage of

spanning the entire membrane, making it several orders of magnitude more stable compared to conventional lipids.

5 Future Directions and Conclusion

The chemistry for functionalizing liposomes with targeting ligands is diverse and has been developed over the last 3 decades. Even so, a number of challenges remain as there is a growing need for reactions that are highly regioselective and efficient. "Click" chemistry is a very good example of the type of chemistry that is required as it provides a very high degree of orthogonality to naturally occurring functional groups. However, the reaction suffers from use of copper as catalyst, and it is not a highly efficient reaction on the surface of liposomes. Another important requirement is that the reactions should be relatively cost efficient where the most important factor is that it proceeds in high yield. Lastly, for *post-functionalization*, the reactions should ideally not give any need for successive purification. Good examples of reactions that fulfill this requirement are the Michael addition to maleimides with thiol-containing ligands and hydrazine condensation with aldehydes that do not require purification if all ligands react. Another important step forward will furthermore be to develop chemistries that are easily evaluated for efficiency as it is not always a trivial task to evaluate reaction progression depending on the ligand being used. This can be envisaged in a number of ways, and it is certain that the coming years will provide much better functionalization chemistries giving high coupling efficiencies, high regio-selectivity and methods for fast evaluation of reaction progression. This is not only for use in drug delivery applications, but also to meet the growing needs in the development of new diagnostic tools where nanoparticles are in increasing demand.

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References

1. Abu Lila AS, Ishida T, Kiwada H (2009) Recent advances in tumor vasculature targeting using liposomal drug delivery systems. Expert Opion Drug Deliv 6:1297–1309
2. Abuchowski A, McCoy JR, Palczuk NC et al (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem 252:3582–3586
3. Agard NJ, Prescher JA, Bertozzi CR (2004) A strainpromoted [3 + 2] azide–alkyne cycloaddition for covalent modification of biomolecules in living systems. J Am Chem Soc 126:15046–15047
4. Albrecht H, Burke PA, Natarajan A et al (2004) Production of soluble ScFvs with C-terminal-free thiol for site-specific conjugation or stable dimeric ScFvs on demand. Bioconjug Chem 15:16–26

5. Allen TM, Brandeis E, Hansen CB et al (1995) A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim Biophys Acta 1237:99–108
6. Allen TM, Sapra P, Moase E (2002) Use of the post-insertion method for formation of ligand-coupled liposomes. Cell Mol Biol Lett 7:889–894
7. Andresen TL, Davidsen J, Begtrup M et al (2004) Enzymatic release of antitumor ether lipids by specific phospholipase A2 activation of liposome-forming prodrugs. J Med Chem 47:1694–1703
8. Andresen TL, Jensen SS, Jørgensen K (2005) Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. Prog Lipid Res 44: 68–97
9. Antony AC (1992) The biological chemistry of folate receptors. Blod 79:2807–2820
10. Baenziger JU, Kornfeld S (1974) Structure of the carbohydrate units of IgA1 immunoglobulin. I. Composition, glycopeptide isolation, and structure of the asparagine-linked oligosaccharide units. J Biol Chem 249:7260–7269
11. Baenziger JU, Kornfeld S (1974) Structure of the carbohydrate units of IgA1 immunoglobufin. II. Structure of the *O*-glycosidically linked oligosaccharide units. J Biol Chem 249:7270–7281
12. Baxter LT, Zhu H, Mackensen DG et al (1994) Physiologically based pharmacokinetic model for specific and nonspecific monoclonal antibodies and fragments in normal tissues and human tumor xenografts in nude mice. Cancer Res 54:1517–1528
13. Béduneau A, Saulnier P, Hindré F et al (2007) Design of targeted lipid nanocapsules by conjugation of whole antibodies and antibody Fab′ fragments. Biomaterials 28:4978–4990
14. Bendas G, Rothe U, Scherphof GL et al (2003) The influence of repeated injections on pharmacokinetics and biodistribution of different types of sterically stabilized immunoliposomes. Biochim Biophys Acta 1609:64–70
15. Binétruy-Tournaire R, Demangel C, Malavaud B et al (2000) Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. EMBO J 19:1525–1533
16. Blume G, Cevc G, Crommelin MDJA et al (1993) Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. Biochim Biophys Acta 1149:180–184
17. Boeckler C, Frisch B, Muller S et al (1995) Immunogenicity of new heterobifunctional cross-linking reagents used in the conjugation of synthetic peptides to liposomes. J Immunol Methods 191:1–10
18. Bonnet D, Ollivier N, Gras-Masse H et al (2001) Chemoselective acylation of fully deprotected hydrazine acetyl peptides. Application to the synthesis of lipopeptides. J Org Chem 66:443–446
19. Bonnet D, Grandjean C, Rousselot-Pailley P et al (2003) Solid-phase functionalization of peptides by an alpha-hydrazinoacetyl group. J Org Chem 68:7033–7040
20. Bonnet L, Pécheur E, Grandjean C et al (2005) Anchorage of synthetic peptides onto liposomes via hydrazone and α-oxo hydrazone bonds. Preliminary functional investigations. Bioconjug Chem 16:450–457
21. Brendas G, Krause A, Bakowsky U et al (1999) Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. Int J Pharm 181:79–93
22. Carrion C, Domingo JC, de Madariaga MA (2001) Preparation of long-circulating immunoliposomes using PEG-cholesterol conjugates: effect of the spacer arm between PEG and cholesterol on liposomal characteristics. Chem Phys Lipids 113:97–110
23. Cavalli S, Tipton AR, Overhand M et al (2006) The chemical modification of liposome surfaces via a copper-mediated [3 + 2] azide–alkyne cycloaddition monitored by a colorimetric assay. Chem Commun 3193–3195

24. Chiu S, Liu S, Perrotti D et al (2006) Efficient delivery of a Bcl-2-specific antisense oligodeoxyribonucleotide (G3139) via transferrin receptor-targeted liposomes. J Control Release 112:199–207
25. Chono S, Tanino T, Seki T et al (2007) Uptake characteristics of liposomes by rat alveolar macrophages: influence of particle size and surface mannose modification. J Pharm Pharmacol 59:75–80
26. Chua M, Fan S, Karush F (1984) Attachment of immunoglobulin to liposomal membrane via protein carbohydrate. Biochim Biophys Acta 800:291–300
27. Ciuchi F, Di Nicola G, Franz H et al (1994) Self-recognition and self-assembly of folic acid salts: columnar liquid crystalline polymorphism and the column growth process. J Am Chem Soc 116:7064–7071
28. Clamp JR, Hough L (1965) The periodate oxidation of amino acids with reference to studies on glycoproteins. Biochem J 94:17–24
29. Crosasso P, Brusa P, Dosio F et al (1997) Antitumoral activity of liposomes and immunoliposomes containing 5-fluorouridine prodrugs. J Pharm Sci 86:832–839
30. Dagar S, Sekosan M, Lee BS et al (2001) VIP receptors as molecular targets of breast cancer: implications for targeted imaging and drug delivery. J Control Release 74:129–134
31. Damodaran D, Jeyakani J, Chauhan A et al (2008) CancerLectinDB: a database of lectins relevant to cancer. Glycoconj J 25:191–198
32. Dawson PE, Muir TW, Clark-Lewis I et al (1994) Synthesis of proteins by native chemical ligation. Science 266:776–779
33. DeFrees SA, Phillips L, Guo L et al (1996) Sialyl Lewis × liposomes as a multivalent ligand and inhibitor of E-Selectin Mediated Cellular Adhesion. J Am Chem Soc 118:6101–6104
34. Derksen JTP, Moselt HWM, Scherphof GL (1988) Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages. Biochim Biophys Acta 971:127–136
35. Duncan RJS, Weston PD, Wrigglesworth R (1983) A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. Anal Biochem 132:68–73
36. ElBayoumi TA, Torchilin VP (2008) Tumor-specific anti-nucleosome antibody improves therapeutic efficacy of doxorubicin-loaded long-circulating liposomes against primary and metastatic tumor in mice. Mol Pharm 6:246–254
37. Elegbede AI, Banerjee J, Hanson AJ et al (2008) Mechanistic studies of the triggered release of liposomal contents by matrix metalloproteinase-9. J Am Chem Soc 130:10639–10642
38. Espuelas S, Haller P, Schuber F et al (2003) Synthesis of an amphiphilic tetraantennary mannosyl conjugate and incorporation into liposome carriers. Bioorg Med Chem Lett 13:2257–2560
39. Espuelas S, Roth A, Thumann C et al (2005) Effect of synthetic lipopeptides formulated in liposomes on the maturation of human dendritic cells. Mol Immunol 42:721–729
40. Fleiner M, Benzinger P, Fichert T et al (2001) Studies on protein–liposome coupling using novel thiol-reactive coupling lipids: influence of spacer length and polarity. Bioconjug Chem 12:470–475
41. Francis GE, Fisher D, Delgado C et al (1998) PEGylation of cytokines and other therapeutic protein and peptides: the importance of biological optimization of coupling techniques. Int J Hematol 68:1–18
42. Frisch B, Boeckler C, Schuber F (1996) Synthesis of short polyoxyethylene-based heterobifunctional cross-linking reagents. Application to the coupling of peptides to liposomes. Bioconjug Chem 7:180–186
43. Gabius H-J (1987) Endogenous lectins in tumors and the immune system. Cancer Invest 5:39-46
44. Gabizon A, Horowitz AT, Goren D et al (1999) Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. Bioconjug Chem 10:289–298

45. Gaertner HF, Offord RE (1996) Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. Bioconjug Chem 7:38–44
46. Gal S, Pinchuk I, Lichtenberg D (2003) Peroxidation of liposomal palmitoyllinoleoylphosphatidylcholine (PLPC), effects of surface charge on the oxidizability and on the potency of antioxidants. Chem Phys Lipids 126:95–110
47. Gao J, Zhong W, He J et al (2009) Tumor-targeted PE38KDEL delivery via PEGylated anti-HER2 immunoliposomes. Int J Pharm 374:145–152
48. Garbuzenko O, Barenholz Y, Priev A (2005) Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. Chem Phys Lipids 135:117–129
49. Garnier B, Bouter A, Gounou C et al (2009) Annexin A5-functionalized liposomes for targeting phosphatidylserine-exposing membranes. Bioconjug Chem 20:2114–2122
50. Geoghegan KF, Stroh JG (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of 2-amino alcohol. Application to modification at N-terminal serine. Bioconjug Chem 3:138–146
51. Ghebremariam B, Sidorov V, Matile S (1999) Direct evidence for the importance of hydrophobic mismatch for cell membrane recognition. Tetrahedron Lett 40:1445–1448
52. Ghose TI, Blair AH, Kulkarni PN (1983) Preparation of antibody-linked cytotoxic agents. Methods Enzymol 93:280–333
53. Graaf AJ, Kooijman M, Hennink WE et al (2009) Nonnatual amino acids for site-specific protein conjugation. Bioconjug Chem 20:1281–1295
54. Gregoriadis G, Wills EJ, Swain CP, Tavill AS (1974) Drug-carrier potential of liposomes in cancer chemotherapy. Lancet 1: 1313–1316
55. Gunawan RC, Auguste DT (2010) The role of antibody synergy and membrane fluidity in the vascular targeting of immunoliposomes. Biomaterials 31:900–907
56. Hansen CB, Kao CY, Moase EH et al (1995) Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. Biochim Biophys Acta 1239:133–144
57. Harding JA, Engbers CM, Newman MS et al (1997) Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes. Biochim Biophys Acta 1327:181–192
58. Hassane FS, Frisch B, Schuber F (2006) Targeted liposomes: convenient coupling of ligands to preformed vesicles using "Click Chemistry". Bioconj Chem 17:849–854
59. Hosokawa S, Tagawa T, Niki H et al (2003) Efficacy of immunoliposomes on cancer models in a cell-surface antigen-density-dependent manner. Br J Cancer 89:1545–1551
60. Huang A, Huang L, Kennel SJ (1980) Monoclonal antibody covalently coupled with fatty acid. J Biol Chem 255:8015–8018
61. Iden DL, Allen TM (2001) In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. Biochim Biophys Acta 1513:207–216
62. Ishida T, Iden DL, Allen TM (1999) A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. FEBS Lett 460:129–133
63. Ishida O, Maruyama K, Tanahashi H et al (2001) Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo. Pharm Res 18:1042–1048
64. Janssen APCA, Schiffelers RM, Hagen TLM et al (2003) Peptide-targeted PEG-liposomes in anti-angiogenic therapy. Int J Pharm 2541:55–58
65. Jensen SS, Andresen TL, Davidsen J et al (2004) Secretory phospholipase A(2) as a tumor-specific trigger for targeted delivery of a novel class of liposomal prodrug anticancer etherlipids. Mol Cancer Ther 3:1451–1458
66. Jolck RI, Berg RH, Andresen TL (2010) Solid-phase synthesis of PEGylated lipopeptides using click chemistry. Bioconjugate Chem 21:807–810
67. Kamps JA, Scherphof GL (1998) Receptor versus non-receptor mediated clearance of liposomes. Adv Drug Deliv Rev 32:81–97

68. Kim S-L, Jeong H-J, Kim E-M et al (2007) Folate receptor targeted imaging using poly(ethylene glycol)-folate: in vitro and in vivo studies. J Korean Med Sci 22:405–411
69. Kinstler OB, Gabriel NE, Farrar CE et al (1999) N-terminally chemically modified protein compositions and methods. US Patent 5:985:265
70. Kirpotin D, Park JW, Hong K et al (1997) Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. Biochemistry 36:66–75
71. Kirpotin DB, Drummond DC, Shao Y et al (2006) Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Res 66:6732–40
72. Koning GA, Morselt HWM, Velinova MJ et al (1999) Selective transfer of a lipophilic prodrug of 5-fluorodeoxyuridine from immunoliposomes to colon cancer cells. Biochim Biophys Acta 1420:153–167
73. Koning GA, Fretz MM, Woroniecka U et al (2004) Targeting liposomes to tumor endothelial cells for neutron capture therapy. Appl Radiat Isot 61:963–967
74. Kung VT, Redemann CT (1986) Synthesis of carboxyacyl derivatives of posphatidylethanolamine and use as an efficient method for conjugation of protein to liposomes. Biochim Biophys Acta 862:435–439
75. Lee C, Barnett J, Reaven PD (1998) Liposomes enriched in oleic acid are less susceptible to oxidation and have less proinflammatory activity when exposed to oxidizing conditions. J Lipid Res 39:1239–1247
76. Lee RJ, Low PS (1994) Delivery of liposomes into cultured KB cells via folate-mediated endocytosis. J Biol Chem 269:3198–3204
77. Lee RJ, Low PS (1995) Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. Biochim Biophys Acta 1233:134–144
78. Lewis WG, Magallon FG, Fokin VV et al (2004) Discovery and characterization of catalysts for azide–alkyne cycloaddition by fluorescence quenching. J Am Chem Soc 126:9152–9153
79. Li Z, Seo TS, Ju J (2004) 1,3-Dipolar cycloaddition of azides with electron-deficient alkynes under mild condition in water. Tetrahedron Lett 45:3143–3146
80. Liang MT, Davies NM, Toth I (2005) Encapsulation of lipopeptides within liposomes: effect of number of lipid chains, chain length and method of liposome preparation. Int J Pharm 301:247–254
81. Lu Y, Low PS (2002) Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv Drug Deliv Rev 54:675–693
82. Maeda H, Wu J, Sawa T et al (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 65:271–284
83. Marsh D (1996) Intrinsic curvature in normal and inverted lipid structures and in membranes. Biophys J 70:2248–2255
84. Martin FJ, Hubbell W, Papahadjopoulos D (1981) Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab′ fragments via disulfide bonds. Biochemistry 20:4229–4238
85. Martin FJ, Papahadjopoulos D (1982) Irreversible coupling of immunoglobulin fragments to preformed vesicles. J Biol Chem 257:286–288
86. Maruyama K, Takizawa T, Yuda T et al (1995) Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim Biophys Acta 1234:74–80
87. Maruyama K, Ishida O, Takizawa et al (1999) Possibility of active targeting to tumor tissues with liposomes. Adv Drug Deliv Rev 40:89–102
88. Matsumura Y, Gotoh M, Muro K et al (2004) Phase I and pharmacokinetic study of MCC-465, a doxorubicin (DXR) encapsulated in PEG immunoliposome, in patients with metastatic stomach cancer. Ann Oncol 15:517–525
89. Meerovitch K, Bergeron F, Leblond L et al (2003) A novel RGD antagonist that targets both alphavbeta3 and alpha5beta1 induces apoptosis of angiogenic endothelial cells on type I collagen. Vasc Pharmacol 40:77–89

90. Mehvar R (2003) Recent trends in the use of polysaccharides for improved delivery of therapeutic agents: pharmacokinetic and pharmacodynamic perspectives. Curr Pharm Biotechnol 4:283–302
91. Menezes DEL, Pilarski LM, Allen TM (1998) In vitro and in vivo targeting of immunoliposomal doxorubicin to human B-cell lymphoma. Cancer Res 58:3320–3330
92. Mercadal M, Domigo JC, Petriz J et al (1999) A novel strategy afford high-yield coupling of antibody to extremities of liposomal surface-grafted PEG chains. Biochim Biophys Acta 1418:232–238
93. Miron T, Wilchek M (1993) A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins. Bioconjug Chem 4:568–569
94. Moghimi SM, Andersen AJ, Hashemi SH et al (2010) Complement activation cascade triggered by PEG-PL engineered nanomedicines and carbon nanotubes: The challenges ahead. J Control Release. doi:10.1016/j.jconrel.2010.04.003
95. Moreira JN, Ishida T, Gaspar R et al (2002) Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. Pharm Res 19:265–269
96. Muñoz M, Garcia M, Reig F et al (1998) Physico-chemical characterization of liposomes with covalently attached hepatitis A VP3(101–121) synthetic peptide. Analyst 123:2223–2228
97. Müller P, Nikolaus J, Schiller S et al (2009) Molecular rods with oligospiroketal backbones as anchors in biological membranes. Angew Chem Int Ed. doi:10.1002/anie.200901133
98. Nässander UK, Steerenberg PA, Jong WHD et al (1995) Design of immunoliposomes directed against human ovarian carcinoma. Biochim Biophys Acta 1235:126–139
99. Ogawa Y, Kawahara H, Yagi N et al (1999) Synthesis of a novel lipopeptide with α-melanocyte-stimulating hormone peptide ligand and its effect on liposome stability. Lipids 34:387–394
100. Pan X, Wu G, Yang W et al (2007) Synthesis of cetuximab-immunoliposomes via a cholesterol-based membrane anchor for targeting of EGFR. Bioconjug Chem 18:101–108
101. Park JW, Kirpotin DB, Hong K et al (2001) Tumor targeting using anti-her2 immunoliposomes. J Control Release 74:95–113
102. Park JW, Hong KL, Kirpotin DB et al (2002) Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. Clin Cancer Res 8:1172–1181
103. Patrick TA, Kranz DM, van Dyke TA et al (1997) Folate receptors as potential therapeutic targets in choroid plexus tumors of SV40 transgenic mice. J Neurooncol 32:111–123
104. Patwardhan AP, Thompson DH (2000) Novel flexible and rigid tetraether cyclic and macrocyclic bisphosphocholines: synthesis and monolayer properties. Langmuir 16:10340–10350
105. Peer D, Karp JM, Hong S et al (2007) Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2:751–760
106. Peeters JM, Hazendonk TG, Beuvery EC et al (1989) Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. J Immunol Methods 120:133–143
107. Reddy JA, Abburi C, Hofland H et al (2002) Folate targeted cationic liposome-mediated gene transfer into disseminated peritoneal tumors. Gene Ther 9:1542–1550
108. Reubi JC (1995) In vitro identification of VIP receptors in human tumors. J Nucl Med 36:1846–1853
109. Reulen SWA, Brusselaars WWT, Langereis S et al (2007) Protein-liposome conjugates using cysteine-lipids and native chemical ligation. Bioconjug Chem 18:590–596
110. Roberts MJ, Bentley MD, Harris JM (2002) Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev 54:459–476
111. Rostovtsev VV, Green LG, Fokin VV et al (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew Chem Int Ed 41:2596–2599

112. Rui Y, Wang S, Low PS et al (1998) Diplasmenylcholine-folate liposomes: an efficient vehicle for intracellular drug delivery. J Am Chem Soc 120:11213–11218
113. Rydzynski K, Jedrychowski R (1994). Sensory irritating properties of cyanuric chloride as revealed with plethysmographic method. Int J Occup Med Environ Health 7:149–154
114. Santos AO, Silva LCG, Bimbo LM et al (2010) Design of peptide-targeted liposomes containing nucleic acids. Biochim Biophys Acta 1798:433–441
115. Sapra P, Allen TM (2003) Ligand-targeted liposomal anticancer drugs. Prog Lipid Res 42:439-462
116. Sapra P, Tyagi P, Allen TM (2005) Ligand-targeted liposomes for cancer treatment. Curr Drug Deliv 2:369–381
117. Saul JM, Annapragada A, Natarajan JV et al (2003) Controlled targeting of liposomal doxorubicin via the folate receptor in vitro. J. Control Release 92:49–67
118. Saxon E, Bertozzi CR (2000) Cell surface engineering by a modified Staudinger reaction. Science 287:2007–2010
119. Schelté P, Boeckler C, Frish B et al (2000) Differential reactivity of maleimide and bromoacetyl functions with thiols: application to the preparation of liposomal diepitope constructs. bioconjugate Chem 11:118–123
120. Schiffelers RM, Koning GA, ten Hagen TLM et al (2003) Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin. J Control Release 93:115–122
121. Schroeder R, London E, Brown D (1994) Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. PNAS 91: 12130–12134
122. Shimada K, Kamps JAAM, Regts J et al (1997) Biodistribution of liposomes containing synthetic galactose-terminated diacylglyceryl-poly(ethyleneglycol)s. Biochim Biophys Acta 1326:329–341
123. Song CK, Jung SH, Kim D-D et al (2009) Disaccharide-modified liposomes and their in vitro intracellular uptake. Int J Pharm 380:161–169
124. Torchilin VP, Goldmacher VS, Smirnov VN (1978) Comparative studies on covalent and noncovalent immobilization of protein molecules on the surface of liposomes. Biochem Biophys Res Commun 85:983–990
125. Torchilin VP, Khaw BA, Smirnov VN et al (1979) Preservation of antimyosin antibody activity after covalent coupling to liposomes. Biochem Biophys Res Commun 89:1114–1119
126. Torchilin VP (1985) Liposomes as targetable drug carriers. Crit Rev Ther Drug Carrier Syst 2:65–115
127. Torchilin VP (2008) TAT peptide-mediated intracellular delivery of pharmaceutical nanocarriers. Adv Drug Deliv Rev 60:548–558
128. Torchilin VP, Levchenko TS, Lukyanov AN et al (2001) *p*-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via *p*-nitrophenylcarbonyl groups. Biochim Biophys Acta 1511:397–411
129. Tornøe CW, Meldal M (2001) Peptidotriazoles: copper(I)-catalyzed 1,3-dipolar cycloadditions on solid-phase. In: Peptides 2001, Proc Am Pept Symp; American Peptide Society and Kluwer Academic Publishers, San Diego, pp 263–264
130. Tornøe CW, Christensen C, Meldal M (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J Org Chem 67:3057–3064
131. Traut RR, Bollen A, Sun TT et al (1973) Methyl 4-mercaptobutyrimidate as a cleavable cross-linking reagent and its application to the *Escherichia coli* 30s ribosome. Biochemistry 12:3266–3273
132. Veronese FM, Largajolli R, Boccu E et al (1985) Activation of monomethoxy poly(ethylene glycol) by phenylchloroformate and modification of ribonuclease and superoxide dismutase. Appl Biochem Biotechnol 11:141–152

133. Vikbjerg AF, Andresen TL, Jørgensen K et al (2007) Oxidative stability of liposomes composed of docosahexaenoic acid-containing phospholipids. J Am Oil Soc 84:631-637
134. Vogel K, Wang S, Lee RJ et al (1996) Peptide-mediated release of folate-targeted liposome contents from endosomal compartments. J Am Chem Soc 118:1581–1586
135. Wang M, Löwik DWPM, Miller AD et al (2009) Targeting the urokinase plasminogen activator receptor with synthetic self-assembly nanoparticles. Bioconjug Chem 20:32–40
136. Weinstein JN, Blumenthal R, Sharrow SO et al (1978) Antibody-mediated targeting of liposomes. Binding to lymphocytes does not ensure incorporation of vesicle contents into the cells. Biochim Biophys Acta 509:272–288
137. Weissig V, Lasch J, Klibanov AL et al (1986) A new hydrophobic anchor for the attachment of proteins to liposomal membranes. FEBS 202:86–90
138. Weitman SD, Weinberg AG, Coney LR et al (1992) Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. Cancer Res 52:6708–6711
139. Woodle MC, Lasic DD (1992) Sterically stabilized liposomes. Biochim Biophys Acta 1113:171–199
140. Wyszynska K, Przybojewska B, Spiechowicz E et al (1994) Cyanuric chloride has no genotoxic and mutagenic properties in bacteria and bone marrow cells. Int J Occup Med Environ Health 7:281–289
141. Xu L, Tang W, Huang C-C et al (2001) Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-Transferrin receptor scFv. Mol Med 7:723–734
142. Xu Z, Jayaseharan JS, Marchant RE (2002) Synthesis and characterization of oligomaltose-grafted lipids with application to liposomes. J Colloid Interface Sci 252:57–65
143. Yagi N, Ogawa Y, Kodaka M et al (2000) Preparation of functional liposomes with peptide ligands and their binding to cell membranes. Lipids 35:673–680
144. Yamada A, Taniguchi Y, Kawano K et al (2008) Design of folate-linked liposomal Doxorubicin to its antitumor effect in mice. Clin Cancer Res 14:8161–8168
145. Zalipsky S (1993) Synthesis of an end-group functionalized polyethylene glycol-lipid conjugate for preparation of polymer-grafted liposomes. Bioconjug Chem 4:296–299
146. Zalipsky S, Puntambekar B, Boulikas P et al (1995) Peptide attachment to extremities of liposomal surface grafted PEG chains: Preparation of the long-circulating form of laminin pentapeptide, YIGSR. Bioconjug Chem 6:705–706
147. Zalipsky S, Mullah N, Dibble A (1999) New chemoenzymatic approach to glyco-lipopolymers: practical preparation of functionally active galactose-poly(ethylene glycol)-distearoylphosphatidic acid (Gal-PEG-DSPA) conjugate. Chem Commun 7:653–654
148. Zhang H, Ma Y, Sun X-L (2009) Chemically-selective surface glyco-functionalization of liposomes through Staudinger ligation. Chem Commun 21:3032–3034
149. Zhu J, Xue J, Guo Z et al (2007) Biomimetic glycoliposomes as nanocarriers for targeting P-Selectin on activated platelets. Bioconjugate Chem 18:1366–1369