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Review

Recent development of poly(ethylene glycol)-cholesterol conjugates as drug delivery systems

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ABSTRACT

Poly(ethylene glycol)-cholesterol (PEG-Chol) conjugates are composed of "hydrophilically-flexible" PEG and "hydrophobically-rigid" Chol molecules. PEG-Chol conjugates are capable of forming micelles through molecular self-assembly and they are also used extensively for the PEGylation of drug delivery systems (DDS). The PEGylated DDS have been shown to display optimized physical stability properties in vitro and longer half-lives in vivo when compared with non-PEGylated DDS. Cell uptake studies have indicated that PEG-Chol conjugates are internalized via clathrin-independent pathways into endosomes and Golgi apparatus. Acid-labile PEG-Chol conjugates are also able to promote the content release of PEGylated DDS when triggered by dePEGylation at acidic conditions. More importantly, biodegradable PEG-Chol molecules have been shown to decrease the "accelerated blood clearance" phenomenon of PEG-DSPE. Ligands, peptides or antibodies which have been modified with PEG-Chols are oftentimes used to formulate active targeting DDS, which have been shown in many systems recently to enhance the efficacy and lower the adverse effects of drugs. Production of PEG-Chol is simple and efficient, and production costs are relatively low. In conclusion, PEG-Chol conjugates appear to be very promising multifunctional biomaterials for many uses in the biomedical sciences and pharmaceutical industries.

Contents

1. Introduction

PEGylation applications are extensively used in the pharmaceutical industry today (Harris and Chess, 2003; Pasut and Veronese, 2009, 2012; Veronese and Pasut, 2005; Wei et al., 2009a), especially for selected drug delivery systems (DDS) in order to improve their physical stability in vitro, to avoid engulfment by mononuclear phagocytes, and to prolong circulation times of novel drugs in vivo (Dufort et al., 2012; Fleige et al., 2012; Otsuka et al., 2003;

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Rodriguez et al., 2013; Torchilin, 2007; Zhao et al., 2013). By "passive" or "active" targeting, PEGylated DDS can accumulate the selected drug cargos specifically at the target site, greatly enhance the efficacy of therapeutic agents, to improve the efficacy of imaging agents, and to lower the toxicity or adverse reactions of drugs resulting in enhanced safety profiles in scientific studies or human clinical trials (Dufort et al., 2012; Ni et al., 2014; Obermeier et al., 2011; Pasut and Veronese, 2009; Peng et al., 2013; Zhu et al., 2013). Cholesterol (Chol, Fig. 1) has been shown to reside in the biological membranes of various organisms, and it can modulate the fluidity of biological membranes involved in many important life events (Rayner et al., 2010). Due to relatively good biocompatibility with the host, as well as low toxicity profiles (Hullin-Matsuda et al., 2009), PEG-Cholesterol (PEG-Chol) conjugates are oftentimes used to modify diverse DDS (PEGylated DDS), including micelles (Li et al., 2012), liposomes (Xu et al., 2008), nanoparticles (Stevens et al., 2004), hydrogels (Rao and Taguchi, 2012; van de Manakker et al., 2009), lipoplexes (He et al., 2013b), and polyplexes (Wang et al., 2007). When combined with the oral or injectable administration of PEG-Chol-based therapeutic agents, PEGylated DDS are now used to treat infectious diseases (Anderson et al., 1999; Liu et al., 2008a), central nervous system diseases (Chen et al., 2012; Yang et al., 2008), augmentation of cancer chemotherapies (Xiong et al., 2011), enhance imaging efficiency in vivo (Pan et al., 2007), and to optimize numerous in vivo gene therapy applications (He et al., 2013a). It is now well known that non-degradable 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-PEG (PEG-DSPE)-modified liposomes or vesicles display "accelerated blood clearance" (ABC) phenomenon (Abu Lila et al., 2013), but biodegradable PEG-Chol conjugates can effectively prolong circulation half-life times of liposomes or vesicles by either limiting or eliminating the ABC phenomenon in vivo (Xu et al., 2010).

This review focuses on PEGylated and dePEGylated DDS by PEG-Chol conjugates (Beugin et al., 1998b; Boomer et al., 2009; Ishiwata et al., 1997; Zhao et al., 2007), and active targeted DDS based on functionalized-PEG-Chol conjugates (Cai et al., 2012; He et al., 2010; Pan et al., 2007). In addition, some novel PEG-Chol conjugates (Hofmann et al., 2010; Rao et al., 2011; van de Manakker et al., 2009) have been reviewed.

2. PEGylation by PEG-Chol in DDS

By using ether (Fig. 2A and B) (Brockerhoff and Ramsammy, 1982; Patel et al., 1984), carbonate (Fig. 2C) (Beugin et al., 1998a,b), carbomate (Fig. 2D) bonds (Bradley et al., 1998) and linkers (Fig. 2E, F and H) (Carrion et al., 2001; Xu et al., 2008; Yang et al., 2008),

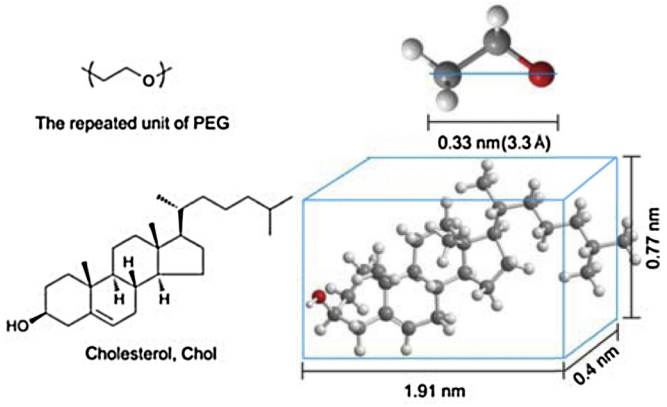


Fig. 1. Structures and dimensions of PEG and Cholesterol. PEG, with a flexible structure, is a hydrophilic molecule (Hansen et al., 2003); Cholesterol (Chol), with a rigid structure, is a hydrophobic molecule (Gimpl and Gehrig-Burger, 2011).

variable molecular weight PEGs are routinely coupled to Chol to synthesize PEG-Chol conjugates. With a hydrophilic PEG "head group", and a hydrophobic Chol segment, PEG-Chol is an amphiphilic surfactant (Beugin et al., 1998a,b). The critical micelle concentration (CMC) of PEG-Chol is $0.4–12.7 \times 10^{-6}$ μM (PEG with molecular weight in the range of 400–10,000 Da) (Buzova et al., 2013; Li et al., 2012; Xu et al., 2005; Yang et al., 2008). Due to the low CMC values like some Poloxamers (Pluronic®) (Alvarez-Lorenzo et al., 2011; Dumortier et al., 2006; Kabanov et al., 2002, 2003), PEG-Chol micelles have high stability and thereby can maintain their integrity even upon strong dilution during systemic circulation (Gong et al., 2012; Han et al., 2009; Li et al., 2012; Liu et al., 2013; Wang et al., 2012; Wei et al., 2009b; Wu et al., 2013). PEG-Chol (Fig. 2B) has been used to prepare a micelle loading Adriamycin (Xu et al., 2005). The Adriamycin-release behavior demonstrated significant sustained release characteristics since it was found that Adriamycin was loaded into the inner core of the micelle. In addition, selected hydrophobic drugs, including Quercetin and Docetaxel (Li et al., 2012; Yu et al., 2013), were solubilized and encapsulated into PEG-Chol molecules (Fig. 2F), and shown to form stable micelle systems. Since the elimination of PEG-Chol (Fig. 2F) under acidic condition (pH 5.0) is enhanced (Xu et al., 2008), these drugs were quickly released in phosphate buffered saline (PBS) at pH 5.0. These micelles appear to be extremely promising vectors for the controlled and targeted drug delivery to several human diseases shown to display acidic microenvironments (Ge and Liu, 2013; Mura et al., 2013). PEG-Chol (Fig. 2A) or PEG-squalene was capable of encapsulating gemcitabine or deoxycytidine and forming nanoparticulate DDS, which displayed superior anticancer activity on gemcitabine-resistant leukemia cell line (Bekkara-Aounallah et al., 2008; Trung Bui et al., 2013).

Historically, PEG-Chol conjugates were originally synthesized to study liposomal bilayer structures (Brockerhoff and Ramsammy, 1982). As rigid structures, the Chol segments of PEG-Chol provide a stabilizing hydrophobic anchor and are therefore able to elevate the "orderliness" (high degree of structural organization) and stability of phospholipids bilayers (Beugin et al., 1998a,b; Brockerhoff and Ramsammy, 1982; Lingwood and Simons, 2010; Patel et al., 1984); PEG segments of PEG-Chol are flexible structures with "steric barrier" properties when anchored in the surfaces of liposomes or niosomes (Dufort et al., 2012). These elevated flexibility properties have several advantages, including: (1) enhancing lipid bilayer impermeability, (2) stabilization of liposomes or niosomes against aggregation, (3) reducing the adsorption of proteins and macromolecules, (4) inhibition of complement activation, (5) increases their stability in buffer systems and human plasma, and (6) prolonging their circulation half-lives (Beugin et al., 1998a,b; Bradley et al., 1998; Janzen et al., 1996; Xu et al., 2008). Additionally, by introducing a "spacer arm", the flexibility of the PEG chain can be reinforced and the rigid Chol segment can then be inserted deeper into the bilayer of liposomes (Carrion et al., 2001). For example, liposomes loaded with calcein were prepared using a cleavable PEG-Chol (Fig. 2F), and the liposome contents were rapidly released under esterase-acid conditions (Xu et al., 2008). By loading niosomes with nimodipine modified by PEG-Chol (Fig. 2G), the results showed a greater accumulative release of drug than that of plain niosomes alone (Yang et al., 2008). When PEG-Chol (Fig. 2A) modified liposomes, designed with a stable size and bilayer impermeability, were loaded with methotrexate, these structures were observed to avoid being taking up quickly by the liver in vivo, which resulted in methotrexate not being leaked into the blood circulation (Patel et al., 1984). This liposome structure significantly extended the survival of mice bearing hepatoma 129 ascites tumors. It is well known that PEG-DSPE-modified liposomes or vesicles possess the

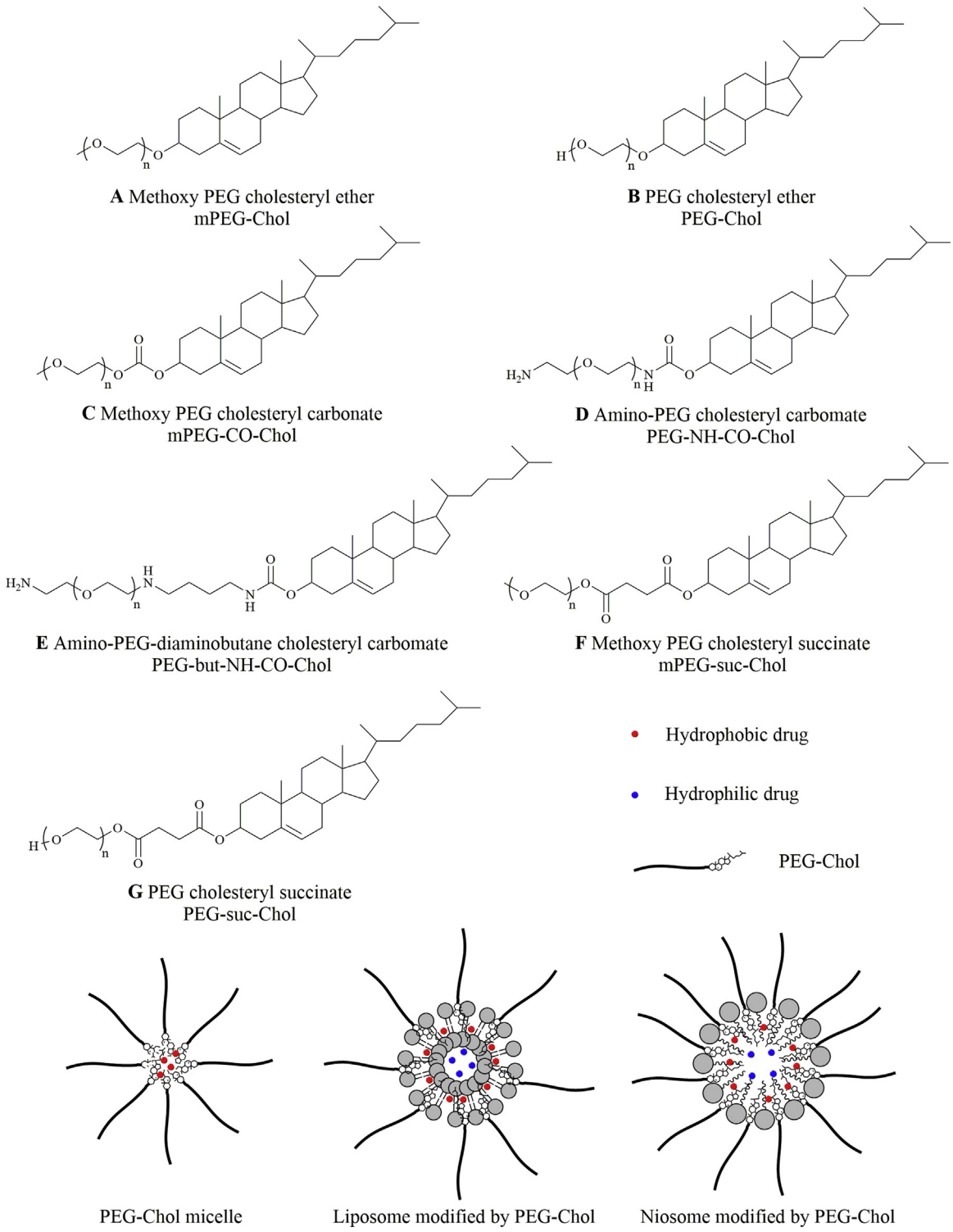


Fig. 2. PEG-Chol conjugate libraries and their application in drug delivery systems. "*n*" is the average repeated number of ethylene glycol units.

ABC phenomenon, but PEG-Chol (Fig. 2C and F) conjugates have been shown to effectively prolong the circulation times of liposomes or vesicles via decreasing or even eliminating the ABC phenomenon in vivo (Abu Lila et al., 2013; Xu et al., 2010).

As a surfactant, PEG-Chol molecules can stabilize cellular lipid bilayers by increasing steric stabilization the low concentrations (for example, 5 mol% for PEG_{2000}-Chol and 10 mol% for PEG_{1000}-Chol). When used as higher concentrations, PEG-Chol can destroy the lipid bilayer and form a hybrid-micelle structure (Beugin et al., 1998a,b).

In recently described studies (Wang et al., 2007), PEG-Chol (Fig. 2B) conjugates have been entrapped into PEI/DNA complexes to improve the stability of the polyplexes in physiological salt concentrations, and it has been observed that PEGylation can significantly improve the stability of these polyplexes which enhances reporter gene transfection efficiency when applied under physiological condition.

PEG derivatives and its preparations (hydrogels, microspheres and micelles) were sterilized by Cobalt-60 irradiation

(Fan et al., 2013, 2014; Fu et al., 2012). In the previous studies, PEG-Chol liposomes were sterilized by microporous membrane filtration method (He et al., 2010), and the well sterile results have been demonstrated in vitro and in vivo studies (He et al., 2013a,b). According to our stability experiments, PEGylated liposomes with PEG-Chol were stable in solution at 4 °C for 1 year (data not shown). DDS loading drug or DNA might be suitable to make a lyophilized preparation (Chen et al., 2010a; Hinrichs et al., 2006; Hinrichs et al., 2005).

3. DePEGylation of acid-labile PEG-Chol in DDS

The process of PEGylation is able to enhance the physical stability of drug conjugates in vitro and to prolong half-lives ($t_{1/2}$) in vivo, however, "PEG brushes" or "PEG clouds" resulting from PEGylation have been shown to act as steric barriers of drug release and cell uptake of PEGylated DDS (Beugin et al., 1998b; Dufort et al., 2012; Ishiwata et al., 1997). It has recently been demonstrated that the dePEGylation process is capable of improving these shortcomings. Price et al. incorporated a pH sensitive cis-aconitic linker into a cholesterol-based PEGylated lipid (Fig. 3A) (Price et al., 2006). The synthesized lipids have also been shown to be acid labile and biodegradable up to 25% within 5 h in PBS of pH 5.0 at 37 °C, compared with ≤1% hydrolysis of the lipids at physiological pH (7.4) and temperature. A hydrazone-based pH-sensitive PEG-Chol conjugate (Fig. 3B), which was observed as being stable at physiological pH (pH 7.4, half-time, 40.9 h), while sensitive to mildly acidic condition (pH 5.5, half-time, 6.7 h), was used to produce pH-sensitive liposomes loaded with Paclitaxel (Chen et al., 2010b). This PEG-Chol conjugate (Fig. 3B) was capable of eliminating the ABC phenomenon induced by repeated injection of PEGylated liposomes by PEG-DSPE (Abu Lila et al., 2013; Chen et al., 2011). These studies indicated that this pH-sensitive liposome was acid-triggered, released more of the model drug, exhibited higher cellular uptake of Paclitaxel and eliminated the ABC phenomenon when compared with conventional liposomes alone. Another acid-cleavable PEG-Chol (Fig. 3C) conjugate has been developed and used to prepare stable PEGylated liposomes loaded with calcein in a physiological buffered solution or cell culture medium with serum (Boomer et al., 2009). Cleavage of PEG-Chol at mildly acidic condition (pH 5.0) resulted in dePEGylation of the latently fusogenic liposomes, thereby triggering the onset of release of its contents. In uptake experiments, the liposomal calcein cargo was directly delivered to the cytoplasm via an acid-triggered dePEGylation and liposome-endosomal membrane fusion process (Boomer et al., 2009). Therefore, the "dePEGylation triggering strategy" appears to be a very promising biomedical formulation process technique for the site-specific cytoplasmic delivery of liposomal contents (Takae et al., 2008).

4. Active targeted drug or gene delivery systems based on PEG-Chol conjugates

It has been conclusively shown that PEGylation is able to enhance the physical stability of numerous therapeutics in vitro and to prolong the half-lives ($t_{1/2}$) of these therapies in vivo (Edwards, 1999; Frishman et al., 2000). "PEG brushes" or "PEG clouds" arising from the PEGylation process can oftentimes pose steric barriers to active and sustained drug release and cell uptake of PEGylated DDS (Beugin et al., 1998b; Ishiwata et al., 1997). In order to improve these PEGylation process shortcomings, it has been demonstrated that numerous ligands, antibodies and/or polypeptides can be conjugated to PEG-Chol molecules and used to prepare highly-efficient active targeted drug delivery systems in humans. Chol is a viable bilayer anchor for the construction of PEGylated (by PEG-Chol) and receptor-targeted (by ligand/antibody-PEG-Chol) liposomes, which have better colloidal stability in vitro and longer circulation half-lives in vivo when compared to

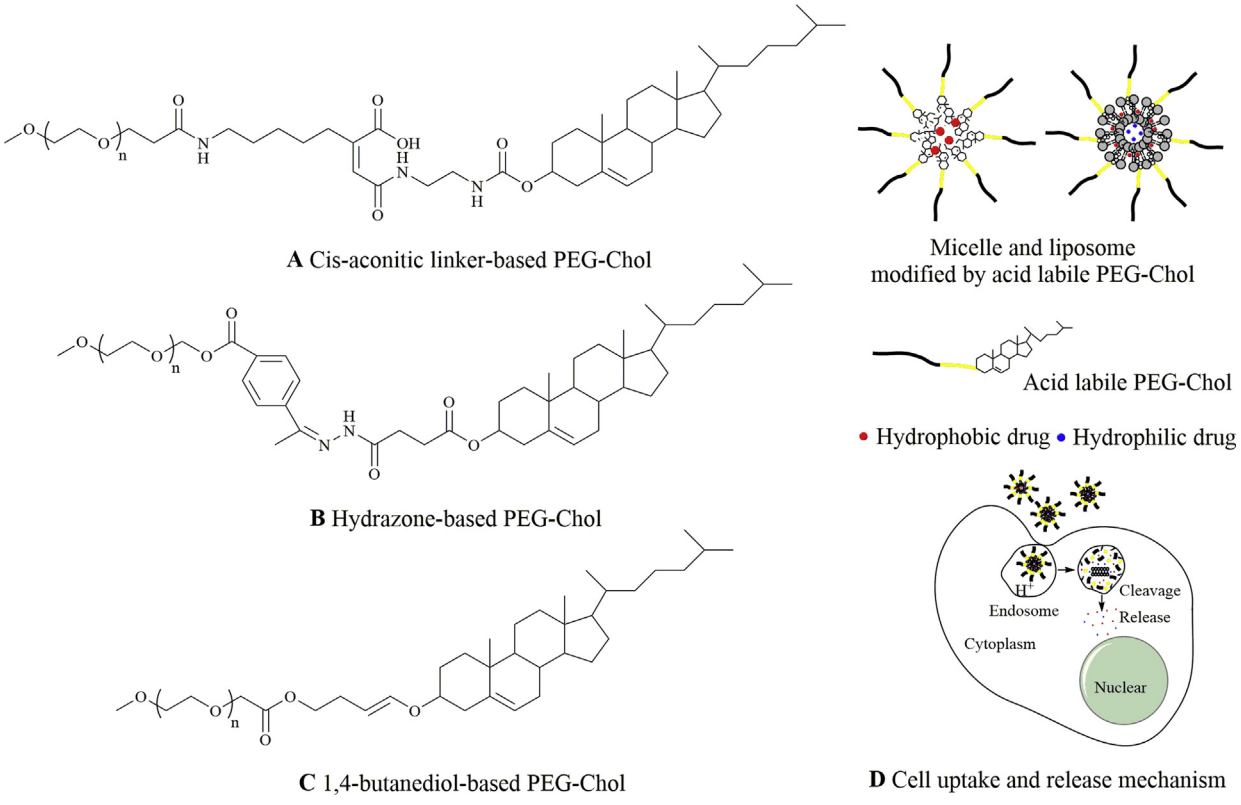
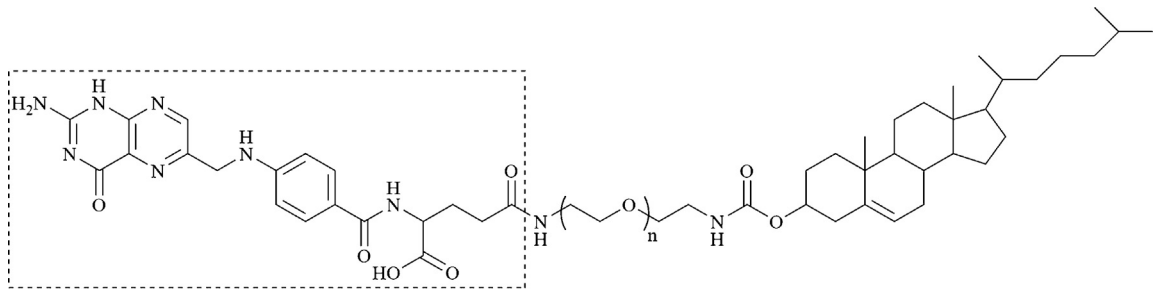
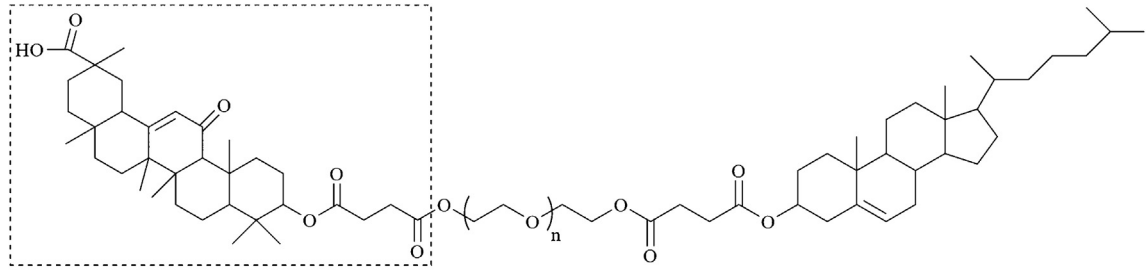


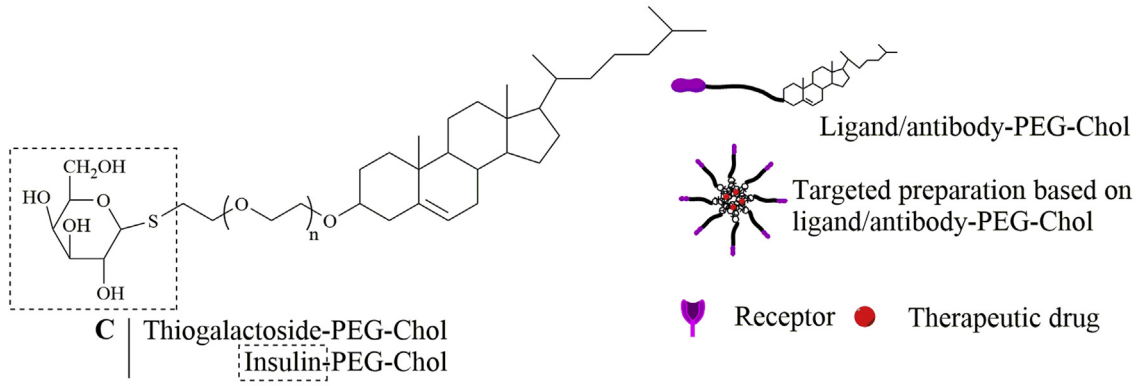
Fig. 3. Acid labile PEG-Chol and dePEGylation in drug delivery systems. "*n*" is the average repeated number of ethylene glycol units.



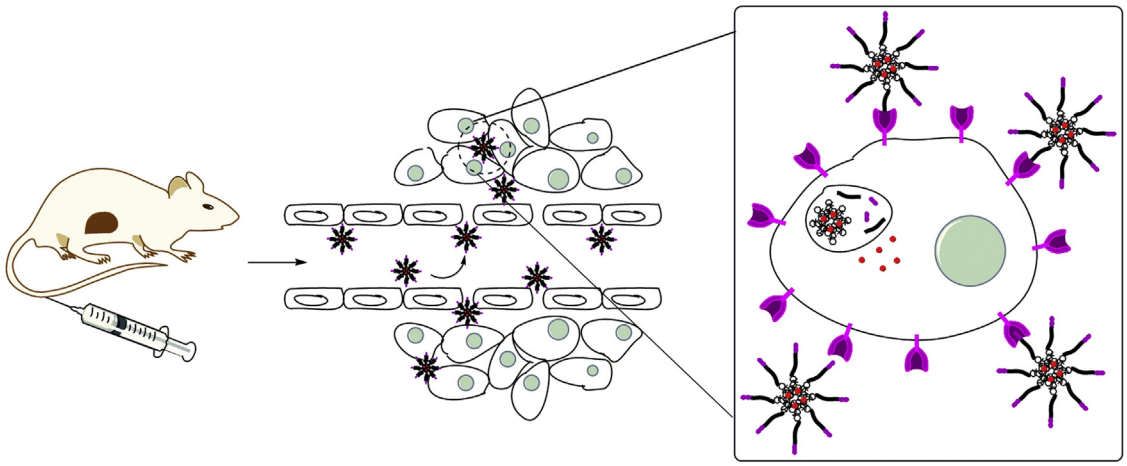
A | Folic Acid / Folate-PEG-Chol
TAT peptide-PEG-Chol
RGD peptide-PEG-Chol
Cetuximab-PEG-Chol



B | Glycyrrhetinic Acid (GA), 3 position hydroxyl or 30 position carboxyl group, GA-PEG-Chol
Folic Acid / Folate-PEG-Chol
Basic fibroblast growth factor fragment (bFGFf)-PEG-Chol



C | Thiogalactoside-PEG-Chol
Insulin-PEG-Chol



D Targeted drug delivery for cancer therapy

Fig. 4. Ligands, antibodies or peptides-PEG-Chol library. Dashed boxes mean replaceable moieties. "*n*" is the average repeated number of ethylene glycol units.

non-PEGylated liposomes (Cai et al., 2012; Chen et al., 2011; Xu et al., 2010). What's more, it has been demonstrated in many systems that receptor-targeted liposomes can be specifically taken up by the interaction between a ligand and receptor (Pan et al., 2007; Zhao et al., 2007). Consequently, different folate-PEG-Chol (Fig. 4A and B) conjugates have been synthesized and used to prepare folate receptor (FR)-targeted liposomes or nanoparticles (Anderson et al., 1999, 2001). Orally FR-targeted liposomes have been shown to improve the apparent permeability of Caco-2 cells (a model gastrointestinal cell line) and increased the uptake and relative bioavailability of poorly absorbed drugs in vitro and in vivo, respectively (Anderson et al., 1999, 2001). FR-targeted and PEGylated liposomes or nanoparticles carrying chemotherapeutic drugs have been shown to increase the uptake and cytotoxicity of tumor cells expressing FR, improve the stability of colloidal systems in vitro, prolong the circulation times, selectively target drugs for tumor cells, to enhance drug accumulation in tumors preclinically, and to enhance the antitumor activity of cytotoxic drugs in tumor-bearing mice resulting in the prevention of potentially dangerous adverse drug side effects (Guo et al., 2000; Stevens et al., 2004; Xiang et al., 2008; Xiong et al., 2011; Yuan et al., 2010; Zhai et al., 2009). For example, Cetuximab was conjugated to PEG-Chol (Fig. 4A) molecules along with synthesizing epidermal growth factor receptor (EGFR) targeted liposomes for the delivery of boron compounds to EGFR(+) glioma cells for neutron capture therapy (Pan et al., 2007). These EGFR targeted liposomes had enhanced cellular uptake of boron compared with nontargeted human IgG-liposomes. Polymeric nanoparticles self-assembled from a trans-activating transcriptional activator peptide-PEG-Chol (TAT-PEG-Col, Fig. 4A) conjugate were fabricated for the delivery of antibiotics across the blood–brain barrier (Liu et al., 2008a,b). The results showed that TAT-nanoparticles were able to cross the blood–brain barrier and specifically target the cell nucleus of neurons. In another example, a truncated basic fibroblast growth factor fragment (bFGFf) was coupled to the distal end of PEG-Chol to produce a bFGFf-PEG-Chol (Fig. 4B), which was respectively used to prepare micelles and liposomes targeting tumor cells which overexpressed fibroblast growth factor receptors (FGFR) (Cai et al., 2011, 2012). bFGFf-modified micelles or liposomes loaded with paclitaxel significantly enhanced the uptake by tumor cells overexpressing FGFR via the interaction between ligand and receptor. The authors concluded that the cytotoxicity of micelles modified by bFGFf was significantly enhanced in vitro. The half-life of paclitaxel in bFGFf-modified liposomes was prolonged in vivo and bio-distribution studies showed high accumulation of bFGFf-modified liposomes in tumor tissue compared with free and non-targeted preparations. It has also been shown that galactoside and insulin grafted onto PEG-Chol conjugates (Fig. 4C) for hepatocyte targeting can be achieved (Hai et al., 2005, 2006; Yang et al., 2010). Glycyrrhetinic acid (GA) was tethered to the terminal of PEG-Chol for obtaining a liver targeting functional-material GA-PEG-Chol (Fig. 4B, the chemical reaction took place at 30 position carboxyl group of GA), which was used to formulate a liver-targeting liposome carrying a model drug referred to as brucine. The sustained targeting of the liver targeting drug delivery GA-modified liposomes was demonstrated by pharmacokinetics studies in rats and tissue distribution studies in mice (Chen et al., 2012).

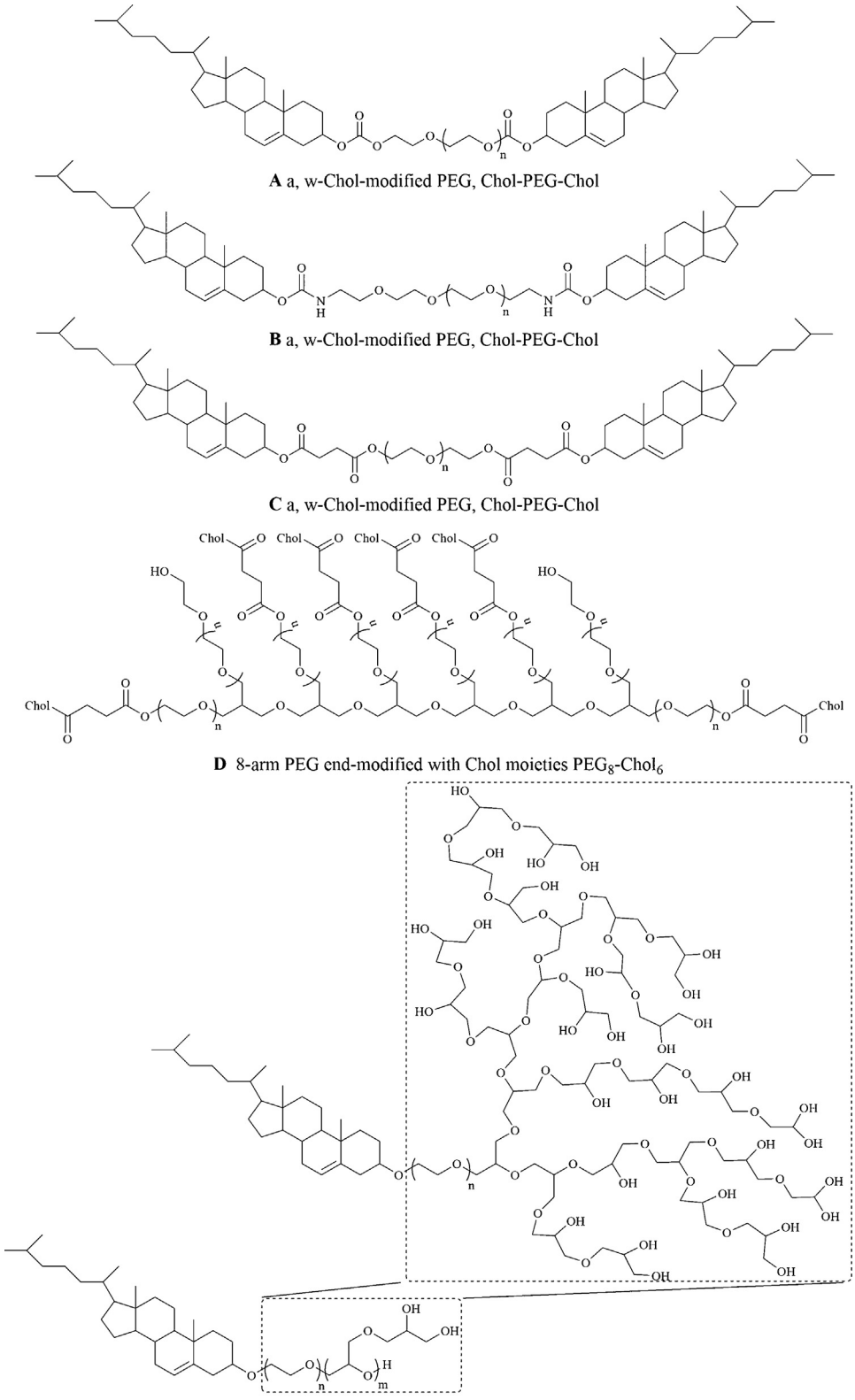
Other GA-PEG-Chol conjugates have been synthesized by the 3′ position hydroxyl group of GA (Fig. 4B), and used to modify cationic liposomes for hepatocyte targeting gene therapy (He et al., 2010). Reports recently published have shown that the gene transfection efficiency of this GA-modified lipoplex was superior to non-targeted PEGylated lipoplexes in vitro. In another report, a RGD peptide was grafted onto PEG-Chol molecules and then encapsulated into preformed liposomes to obtain "neutral post grafted lipoplexes", which inhibited the nonspecific cell binding of conventional lipoplexes with a positive charge (Thompson et al., 2005). Compared to PEGylated cationic lipoplexes, the RGD-modified lipoplex decreased the accumulation of active drug in the lung and achieved tumor targeting in vivo. Folate-PEG-Chol (Fig. 4B) has also been used to synthesize an ovarian cancer

Table 1
Research of PEG-Chol conjugates modified DDS along with the in vivo results.

PEG-Chol conjugates	DDS	Administration route	Outcome	References
PEG-Chol	Liposomes loading methotrexate	Intravenous and intraperitoneal	The liposomes were able to avoid being taking up quickly by the liver and extended the survival of mice bearing tumors.	(Patel et al., 1984)
PEG-Chol	Liposomes loading calcein	Intravenous	The cleavable PEG-Chol could eliminate the ABC phenomenon produced by repeated injection of PEGylated liposomes or vesicles.	(Chen et al., 2011; Xu et al., 2008; Xu et al., 2010)
Folate-PEG-Chol	Liposomes loading doxorubicin, daunorubicin or docetaxel	Intravenous	The liposomes decreased the biodistribution of drug in the heart, brain and kidneys, extended the $t_{1/2}$ and enhanced tumor accumulation and antitumor activity of drug.	(Guo et al., 2000; Xiang et al., 2008; Xiong et al., 2011; Yuan et al., 2010; Zhai et al., 2009)
Folate-PEG-Chol	Liposomes loading vancomycin	Oral	The liposomes resulted in a dramatic increase in the oral delivery of drug.	(Anderson et al., 2001)
Folate-PEG-Chol	Nanoparticles loading paclitaxel	Intraperitoneal	The nanoparticles resulted in significantly greater tumor growth inhibition and animal survival compared to controls.	(Stevens et al., 2004)
RGD-PEG-Chol	Lipoplexes loading reporter gene	Intravenous	Lipoplexes postgrafted by RGD-PEG-Chol decreased the accumulation in the lung and achieved the result of tumor targeting.	(Thompson et al., 2005)
TAT-PEG-Chol	Nanoparticles and micelles loading ciprofloxacin	Intravenous	The nanoparticles were able to cross the blood–brain barrier, and located around the cell nucleus of neurons.	(Liu et al., 2008a,b)
GA-PEG-Chol	Liposomes loading brucine	Intravenous	The liposomes were able to improve the liver targeting of brucine.	(Chen et al., 2012)
bFGF-PEG-Chol	Liposomes loading paclitaxel	Intravenous	The liposomes showed high accumulation of drug in tumor tissue, liver and spleen, but a considerable decrease in other organs (heart, lung and kidney).	(Cai et al., 2012)
Folate-PEG-Chol	Lipoplexes loading shRNA	Intraperitoneal	The lipoplexes promoted benign differentiation of tumor and achieved about 90% tumor growth inhibition.	(He et al., 2013a)

targeted gene delivery system (He et al., 2013b). Folate-modified lipoplexes loaded with shRNA targeting claudin3 could enhance gene delivery in vivo (He et al., 2013a). After gene therapy, claudin3 protein production and gene expression was almost completely down-regulated and ovarian tumor growth was significantly inhibited.

As shown in Table 1, Some reports have demonstrated that DDS modified by PEG-Chol conjugates were able to avoid being taking up quickly by the liver (Patel et al., 1984), decrease the accumulation in the heart, brain, lung and kidney (Cai et al., 2012; Thompson et al., 2005; Yuan et al., 2010), eliminate the ABC phenomenon (Chen et al., 2011; Xu et al., 2008, 2010), extend the



A a, w-Chol-modified PEG, Chol-PEG-Chol

B a, w-Chol-modified PEG, Chol-PEG-Chol

C a, w-Chol-modified PEG, Chol-PEG-Chol

D 8-arm PEG end-modified with Chol moieties PEG_8-$Chol_6$

E Chol-initiated PEG-hyperbranched polyglycerol block copolymer (Chol-PEG-hbPG)

Fig. 5. Novel PEG-Chol conjugates. "*n*" is the average repeated number of ethylene glycol units.

half-life and enhance tumors or target organs accumulation (Anderson et al., 2001; Cai et al., 2012; Chen et al., 2012; Guo et al., 2000; He et al., 2013a; Liu et al., 2008a,b; Stevens et al., 2004; Thompson et al., 2005; Xiang et al., 2008; Xiong et al., 2011; Yuan et al., 2010; Zhai et al., 2009).

5. Novel PEG-Chol conjugates in DDS

Some novel and chemically complex PEG-Chol conjugates have been synthesized (Fig. 5) and applied to various drug delivery systems. For example, α,ω-cholesterol-modified PEGs (Chol-PEG-Chol) have been synthesized using carbonate and amide bond chemistry (Fig. 5A or B) (Meier et al., 1996; Rao et al., 2011). Chol-PEG-Chol molecules functionally insert Chol into the hydrophobic lipid bilayers of liposomes or live cell membranes (Rao and Taguchi, 2012). Chol-PEG-Chol with higher concentrations have been shown to "interconnect" or "bridge" different vesicles or biological cells which leads to the formation of liposomal hydrogels or blood coagulation (Meier et al., 1996). Furthermore, Chol-PEG-Chol can flocculate nanoparticles imprinted by Chol, as demonstrated by the formation of a three-dimensional network of Chol-PEG-Chol molecules and nanoparticles (Perez et al., 2001). A liposomal gel exhibited temperature stimuli responsive behavior and thermo-reversibility by adding Chol-PEG-Chol to a liposome solution (Rao et al., 2011). The zeta potential of cationic liposomes modified by Chol-PEG-Chol (Fig. 5C) molecules has been shown to be decreased when compared to cationic liposomes without Chol-PEG-Chol molecules; the result was that the non-specific binding and transfection efficiency were greatly reduced in vitro (Ma et al., 2014). Based on the formation of β-cyclodextrin (βCD)/Chol inclusion complexes, fully thermo-reversible and elastic hydrogels were formed after hydration of a mixture of star-shaped 8-arm PEG (PEG_8) end-modified with βCD groups (PEG_8-$βCD_8$), and the same PEG_8 end-modified with Chol moieties (PEG_8-$Chol_6$, Fig. 5D) (van de Manakker et al., 2008, 2010). Hydrogel degradation was mainly the result of surface erosion, which depended on the network swelling stresses and initial crosslink density of the gels (van de Manakker et al., 2009). The protein releasing behavior of these hydrogels showed that the degradation mechanism led to a quantitative and nearly zero-order release of entrapped proteins (van de Manakker et al., 2009). Based on linear PEG and hyper-branched polyglycerol (hbPG) (Fig. 5E), Chol-PEG-hbPGs can be synthesized and used to prepare unilamellar liposomes in the size ranges of 40–50 nm (Hofmann et al., 2010).

6. The interaction mechanism between PEG-Chol and cell

In order to study the interaction mechanism between PEG-Chol and cell, the fluorescently tagged-PEG-Chol conjugates have been designed and synthesized as seen in Fig. 6A–C. Free Chol can be inserted to the cell surface and "flip-flop" rapidly in a model membrane; however, the biological properties of PEG-Chol are different from free Chol (Gimpl and Gehrig-Burger, 2011; Wustner, 2007). PEG-Chol can insert itself into a cell membrane by the Chol segment, but the bulk PEG moiety of PEG-Chol prevents trans-bilayer movement of the molecule (Hullin-Matsuda et al., 2009). It has been observed that PEG-Chol distributed exclusively to the exoplasmic leaflet (Sato et al., 2004). This process inhibits the transport of PEG-Chol to the cytoplasmic leaflet and the nonspecific diffusion of the molecule to the cytoplasm. Under low levels of PEG-Chol molecules and short incubation times, it is possible to follow the fate of cell surface PEG-Chol in living cells (Fig. 6D, left panel). With higher concentrations and prolonged incubation times, PEG-Chol molecules binding to the surfaces of mammalian cells are slowly internalized via clathrin-independent pathway into endosomes, and they can further be transported to the Golgi apparatus (Fig. 6D, right panel) (Hullin-Matsuda et al., 2009).

PEGylated liposomes can be obtained when liposomes are modified by PEG-Chol molecules, due to the bulk of the PEG moiety on the surface of liposomes; the internalization of PEGylated liposomes can be drastically reduced in living cells (Ishiwata et al., 1997). When PEG-Chol molecules from liposomes was further encapsulated into the plasma membrane, it was observed that PEG-Chol could reduce the membrane dynamics essential for clathrin-independent invagination of the membrane and to inhibit the endocytic process of liposomes at the cellular level (Hullin-Matsuda et al., 2009). Therefore, the "double activity" of the PEG-Chol conjugate, one at the liposomal level (PEGylated liposomes), obtained by the coating with a hydrophilic and steric stabilized barrier, and the other at the cellular level by slowing down the endocytic process, can significantly enhance the possibility of liposomes modified by PEG-Chol to have long circulation times (Hullin-Matsuda et al., 2009; Ishiwata et al., 1997).

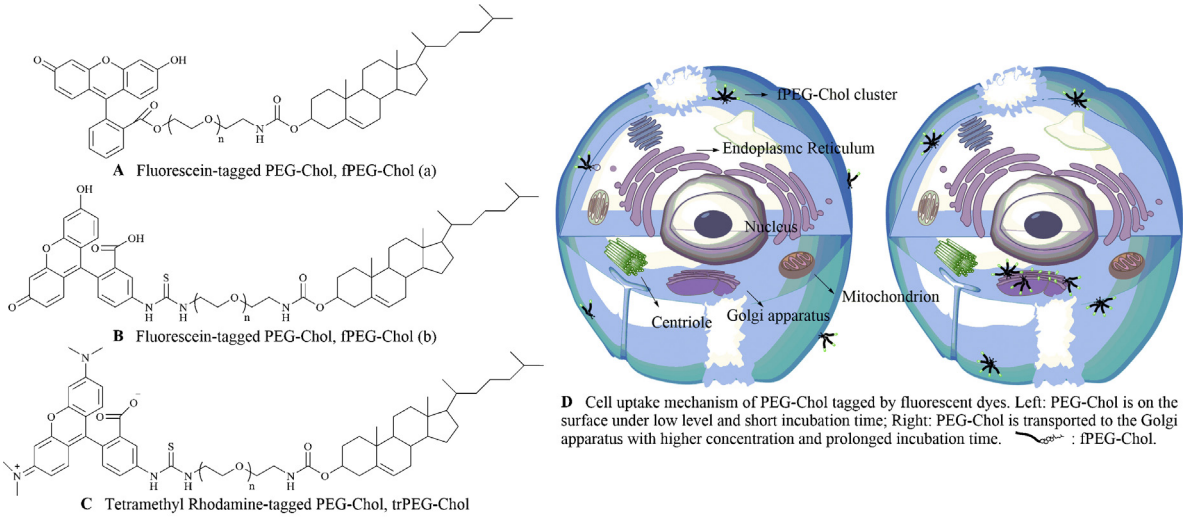


Fig. 6. The fluorescently tagged-PEG-Chol (A, B and C) and interaction mechanism between PEG-Chol and cells (D). "*n*" is the average repeated number of ethylene glycol units.

7. Prospects

PEG-Chol conjugates with low CMC values like PEG-DSPE have been used to prepare PEG-Chol micelles with high drug encapsulation and stability in vitro and in vivo. PEG-Chol conjugates have been shown to be a superior lipid anchor when compared with PEG-DSPE (Abu Lila et al., 2013; Xu et al., 2008, 2010). The PEG segment is anchored effectively into the lipid bilayer or hydrophobic structure through the Chol moiety. PEGylated DDS based on PEG-Chol molecules have been shown to have more physical stability in vitro and longer half-lives in vivo. More importantly, PEG-Chol conjugates are able to lessen or even diminish the ABC phenomenon observed with PEG-DSPE molecules, and then PEG-Chol conjugates are potential for replacing PEG-DSPE in DDS in order to repeated intravenous administration. Furthermore, when PEG-Chol molecules are modified by ligands, peptides or antibodies, they are capable of actively targeting cells or organs specifically expressing receptors or antigens. Considering the many advantages of PEG-Chol formulation chemistry versus PEG-DSPE formulation chemistry, combined with its low cost and feasibility of commercial manufacturing, PEG-Chol conjugates appear to have great promise to actively in the pharmaceutical and biomedical industries.

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