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Effect of Surface Properties on Liposomal siRNA Delivery

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Abstract

Liposomes are one of the most widely investigated carriers for siRNA delivery. The surface properties of liposomal carriers, including the surface charge, PEGylation, and ligand modification can significantly affect the gene silencing efficiency. Three barriers of systemic siRNA delivery (long blood circulation, efficient tumor penetration and efficient cellular uptake/endosomal escape) are analyzed on liposomal carriers with different surface charges, PEGylations and ligand modifications. Cationic formulations dominate siRNA delivery and neutral formulations also have good performance while anionic formulations are generally not proper for siRNA delivery. The PEG dilemma (prolonged blood circulation vs. reduced cellular uptake/endosomal escape) and the side effect of repeated PEGylated formulation (accelerated blood clearance) were discussed. Effects of ligand modification on cationic and neutral formulations were analyzed. Finally, we summarized the achievements in liposomal siRNA delivery, outlined existing problems and provided some future perspectives.

Keywords

siRNA delivery; liposomes; surface charge; PEGylation; ligand modification

1. Introduction

1.1 Challenges of siRNA delivery

RNA interference (RNAi) [1] is a biological process such that the expression of mRNA is inhibited (*i.e.*, silenced) by short double-stranded RNAs (dsRNAs) [2]. In an RNAi, short dsRNAs dissociate to release passenger strand RNAs which could bind to a protein complex named RNA-induced silence complex (RISC). RISC then binds to its complementary mRNA and cuts the binding sequences so that the expression of target mRNA is inhibited.

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By adopting RNAi technology, RNAi therapeutics could target and downregulate genes of interest, which provides a promising way to treat a wide range of genetic diseases and cancers [3]. There are two main types of short dsRNAs involved in RNAi therapeutics: endogenous *microRNAs* and exogenous *small interfering RNAs* (siRNAs). Most microRNAs [4] come from natural RNA segments in nature cells, while siRNAs are artificially designed and synthesized. On the other hand, since the sequence of siRNA can vary according to the sequence of the target mRNA, siRNAs can silence various genes of interest upon proper design. This feature is indeed favorable for cancer therapies. Actually, many functional siRNAs have been designed to target both oncogenes and other key genes that are involved in cancer cell proliferation, survival, invasion, angiogenesis, metastasis, and resistance to chemotherapy and radiotherapy [5, 6]. Furthermore, siRNA cocktails, which contain multiple types of therapeutic siRNAs, are more potent in inhibiting the functions of target mRNAs and proteins than individual siRNA since they can simultaneously silence multiple genes.

Due to their particular structures, however, siRNAs cannot be directly used in nucleic acid therapy. First, siRNAs are not stable and could be digested by endonuclease in the serum (with a half-life of only 15 min [7]). Second, most siRNAs are anionic (*i.e.*, negatively charged) and hence have quite low cellular uptake due to their electrostatic repulsion against negatively charged cell membranes. Chemical modifications can partially solve the above problems. For example, 2-O-methylated siRNAs are resistant to endonuclease, and some neutrally charged peptide nucleic acids (PNAs) [8] could have a better cellular uptake. Furthermore, GalNac conjugated siRNAs developed by Alnylam Pharmaceticals have both high stability and high cellular uptake [9]. Even so, potent gene carriers are still needed to transport siRNAs into target cells for therapeutic use. Besides its stability in serum (*e.g.*, resisting endonuclease), an ideal siRNA carrier should have the following three key features for efficient systemic siRNA delivery [3]:

- *Prolonged blood circulation time*: siRNA carriers as well as siRNAs should have sufficiently long circulation time for effective tumor accumulation;
- *Efficient penetration into tumor tissues*: gene carriers should be able to pass through the vascular endothelium and the extracellular matrix to reach tumor tissues;
- High cellular uptake and efficient endosomal escape: gene carriers should have sufficiently high cellular uptake and efficient endosomal escape to allow siRNAs to release from the endosome, resulting in high cytoplasm delivery.

1.2 Liposomes for siRNA delivery

To overcome the challenges of systemic siRNA delivery, liposomes [10-17], cationic polymers [18], and inorganic nanoparticles [19] (gold, silica, quantum dots, calcium phosphate, *etc.*) have been studied as siRNA carriers. Among them, liposomes are the most widely studied gene carriers due to their excellent biodegradability and biocompatibility. Many liposomal siRNA delivery formulas, such as ALN-VSP02 (targeting *KSP* and *VEGF* genes), siRNA-EphA2-DOPC (targeting *EphA2* gene), and Atu027 (targeting *PKN3* gene), are already in clinical trials [16].

In the liposomal siRNA delivery system, many parameters would affect the gene delivery efficiency. These parameters include the fusogenicity of liposomes, the size of liposomes, the type of surface charge, the degree of PEGylation, the structure of targeting ligands, the type of siRNAs and the location of targeting tissues, and so on. There are already a number of excellent reviews on liposomal formulations, which focus on the efficacy [10, 11, 16, 17], stability [12], chemical structures of lipids [14], nucleic acid therapy applications [15], and clinical trials [5]. In this review, we would like to emphasize the surface properties of liposomes in the liposomal siRNA delivery system. These surface properties include surface charge, PEGylation, and surface targeting ligands [20].

This review is arranged as follows: firstly, we will introduce four physical structures of liposomal carriers for siRNA delivery, which have different surface properties; next, we will discuss the effect of surface charge of liposomal formulation on siRNA delivery, mostly focus on cationic formulations; then we will analyze the effect of PEGylation on siRNA delivery; finally, we will analyze the effect of ligand modification on cationic and neutral formulations. In this way, we summarize the success researchers have achieved in liposomal siRNA delivery and outline the existing problems.

2. Liposome structures for siRNA delivery

Liposomal siRNA carriers can be categorized into four main types by their physical structures, namely, lipoplexes, stable nucleic-acid-lipid particles (SNALPs), lipopolyplexes, and membrane/core nanoparticles (MCNPs) (Figure 1).

2.1 Lipoplexes

Lipoplexes (Figure 1a) generally have multilamellar structures, which are verified by TEM images [21]. In a lipoplex, lipids form multiple bilayers and siRNAs are embedded between adjacent lipid bilayers. Lipoplexes can only be formed from cationic liposomes.

Lipoplexes are easy to synthesize by simply mixing cationic liposomes and siRNAs at expected ratios [22]. In this process, siRNAs first induce the aggregation of liposomes and the rupture of lipid membranes, and then the ruptured membranes wrap around the siRNA-coated liposomes to form multilamellar structure [21]. The synthesis of lipoplexes is convenient and robust. Several commercially available cationic liposome formulations such as Lipofectin, Lipofectamine, and LipoRNAiMAX, form efficient lipoplexes and are often used in *in vitro* gene delivery studies [22].

2.2 SNALPs

In SNALPs (Figure 1b), siRNAs are loaded in the interior of liposomes (close to the inner membrane due to the electrostatic attraction) [23] with high efficiency. Different from lipoplexes, the surface charge of SNALPs is nearly neutral. Some SNALPs are already undergoing tests in clinical trials [16].

Only liposomes containing acidic pH-sensitive lipids (*e.g.*, cationic at pH = 4 and neutral at pH = 7.4) can form SNALPs. The pH-sensitive lipids play an important role in the synthesis of SNALPs. A typical procedure to synthesize SNALPs is as follows [12]:

(1) Liposome preparation: inject ethanol solution of lipids into an acidic buffer (pH = 4). The liposomes obtained have high membrane permeability due to the presence of ethanol and are cationic at such a low pH.

(2) siRNA loading: mix the preformed liposome dispersion with siRNA solution (dispersed in the same acidic buffer). Due to the membrane permeability, siRNAs can penetrate into the interior of the liposomes and bind to the internal surface by electrostatic interactions.

(3) Removal of unencapsulated siRNAs: dialyze the mixture against neutral buffer (pH = 7.4). After dialysis, the external surface of the liposomes becomes neutral and the residual unencapsulated siRNAs can be released.

2.3 Lipopolyplexes

Lipopolyplexes (Figure 1c) are liposomes containing polyplexes (cationic-polymer/siRNA complexes). The lipopolyplex concept was borrowed from plasmid DNA delivery by liposomes [24].

Lipopolyplexes are synthesized by coating the cationic lipid bilayer onto anionic polyplexes [25] or by coating the anionic lipid bilayer onto cationic polyplexes through electrostatic attraction, with the latter being less used. PEGylation is often needed to increase the stability for both *in vitro* and *in vivo* applications. Alternatively, lipopolyplexes can also be synthesized by direct hydration of lipid membranes with polyplex dispersions (*e.g.* multifunctional envelope-type nanodevice (MEND) [26]). A typical synthetic procedure of MEND is as follows [26]: (1) cationic polyplexes are formed by mixing siRNA and stearyl-octaarginine in proper ratios; (2) the cationic polyplex dispersion is added to cationic or neutral dry-lipid-membrane and the mixture is then sonicated to form lipopolyplexes.

2.4 MCNPs

A membrane/core nanoparticle (MCNP), illustrated in Figure 1d, has a core-shell-like structure with one or more inorganic nanoparticles as the core and a lipid bilayer as the shell. The core could be porous calcium phosphate (CaP) or silica (SiO_2) nanoparticles, which can load high amount of siRNAs on their porous surfaces.

In an MCNP, lipid bilayer could be either symmetric (neutral bilayer or cationic bilayer) [27] or asymmetric (anionic inner-layer and cationic outer-layer) [28]. MCNPs with symmetric bilayers are generally synthesized in two steps [27, 29]: firstly, inorganic NPs are synthesized and siRNAs are loaded onto the porous surface of NPs; secondly, lipid bilayers are coated onto the preformed inorganic NPs though electrostatic attraction. For asymmetric MCNPs [28], the synthetic procedure is similar except that inner-layer and outer-layer are coated sequentially. In addition, symmetric MCNPs can also be synthesized by *in situ* mineralization of CaP in the interior of preformed siRNA-loaded liposomes [30].

Since the liposome structure is supported by the inner solid nanoparticle core, MCNPs are more stable than hollow liposomes. In some circumstances, the size of MCNPs depends on the size of the core nanoparticles and MCNPs can be very small (~30 nm) [28]. A smaller size of gene carriers can be beneficial in penetrating tissues and in the cellular uptake,

leading to a higher gene silencing efficiency. However, the synthesis of MCNPs is rather complex, due to the need of co-synthesis of inorganic NPs.

3. The effect of surface charge on liposomal siRNA delivery

In this section we focus on the effect of the surface charge of liposome carriers involved in siRNA delivery. As discussed in Section 1, effective systemic siRNA delivery requires: (1) sufficiently long blood circulation time, (2) efficient penetration into tumor tissues, and (3) high cellular uptake and efficient endosomal escape. In the liposomal siRNA delivery platform, it has been realized that the surface charge of liposomes can have a profound effect on siRNA delivery as it affects all the three processes.

In the blood circulation stage, both cationic and anionic liposome carriers have a relatively short circulation time. On the contrary, neutral liposome carriers are relatively stable and the circulation time is longer than that of charged liposomes [31]. In the tumor penetration stage, neutral liposomes are also more efficient than cationic and anionic ones [32]. However, during the cellular uptake stage and endosomal escape stage of cancer cells, cationic formulations (*i.e.*, cationic liposome carriers) have a better performance than neutral ones, and anionic formulations have the least efficiency [33].

3.1 Cationic liposomes

In general, there are four types of cationic liposomal carriers for siRNA delivery, which are monovalent cationic liposomes, pH-sensitive cationic liposomes, lipidoids, and multivalent cationic liposomes. It is worth noting that blood circulation of cationic liposomes is poor due to the positive surface charge, which increases their complement activation [34] and macrophage uptake [33]. Also, the poor tumor penetration of cationic liposomes impedes their application *in vivo*. Therefore, here we focus on the *in vitro* gene delivery of these cationic liposomes in this subsection.

3.1.1 Monovalent cationic liposomes—The most representative monovalent cationic lipids used in siRNA delivery are DOTMA and DOTAP (Figure 2a). Their head groups contain a strong-cationic quaternary ammonium group, hence DOTMA (or DOTAP) incorporated liposomes have cationic surfaces. These cationic liposomes can form lipoplexes, lipopolyplexes, or MCNPs. The commercially available transfection agent Lipofectin contains 1:1 ratio of DOTMA and DOPE. The Lipofectin/siRNA lipoplexes strongly inhibit the *HIV* replication by silencing *HIV* genes *in vitro* [35]. DOTMA based lipopolyplexes have a similar efficiency as Lipofectamine, knocking down 80% of luciferase at 100 nM of anti-luciferase siRNA *in vitro* [36].

Besides DOTMA and DOTAP, there are also other monovalent cationic lipids such as DC-Chol and DDAB. DC-Chol-based lipoplexes have a lower gene silencing efficiency than that of DOTMA and DOTAP: 20% HER2 gene knockdown at a dose of 0.5 μg siRNA per well (48-well plate) [37]. DDAB-based lipoplexes are shown to have similar gene silencing efficacy as DOTAP-based lipoplexes *in vitro* [38].

Lipoplexes formed from monovalent cationic liposomes usually have a high gene silencing efficiency *in vitro*. There are two reasons. First, they have very high cellular uptake. While there is no literature specifically analyzing the interaction between siRNA/liposome lipoplexes and cell membrane, we still can get some insights from studies on DNA/liposome lipoplexes. It was found that DNA/liposome lipoplexes bind to cell membrane through nonspecific ionic interaction and the proteoglycans on the cell membrane plays an important role in cellular uptake process [39-41]. Second, they have efficient endosomal escape by the ion-pair mechanism [15, 42]. The cationic lipids in the lipoplexes first form ion pairs with anionic lipids (such as phosphatidylserine) from the endosome membrane; and then the ion-pairs lead to disassembly of lipoplexes and allow siRNAs to release from lipoplexes; meanwhile, the ion-pairs also promote the formation of the inverted hexagonal phase (H_{II}) in the binding lipids and trigger the membrane fusion between lipoplexes and endosome membrane, leading to destablization of endosome membrane; finally, the free siRNAs can successfully escape from endsomes.

Lipopolyplexes formed from monovalent cationic liposomes even have a better stability and higher gene transfection efficiency than lipoplexes *in vitro*. While lipopolyplexes with similar surface as lipoplexes have similar cellular uptake, they have more efficient endosomal escape of free siRNAs, since the cationic polymers in lipopolyplexes enhance the endosomal escape by the proton sponge effect [42, 43]. Specifically, cationic polymers in the polyplexes are weak bases and can bind to a lot of protons (so-called proton sponge) in the acidic endosomes, thus increase the osmotic pressure in the endosomes, leading to disruption of endosomes and release of siRNAs.

3.1.2 pH-sensitive cationic liposomes—Different from monovalent cationic lipids, the pH-sensitive ones are neutral at physiological pH and cationic (protonated) at acidic pH. Therefore, at neutral pH, the pH-sensitive cationic liposomes can only form SNALP structures, but not lipoplexes or lipopolyplexes. A representative synthesis procedure of SNALPs can be found in Section 2.2. Figure 2b shows the chemical structures of common pH-sensitive cationic lipids: DLinDMA, DLin-KC2-DMA and YSK05 lipid. They all contain one protonable ternary amine group. Since all the pH-sensitive cationic liposomes are PEGylated, their properties will be discussed in detail in Section 4.2.1.

3.1.3 Lipidoid nanoparticles—The structure of lipidoid (Figure 2c) is similar to that of pH-sensitive lipid but with some differences. In a lipidoid, the head group has more than one site that can be protonated, and the hydrophobic part consists of more than two tails.

Like pH-sensitive lipids, lipidoids are also pH-sensitive. Hence, lipidoid nanoparticles (lipidoid-based liposomes) for siRNA delivery also have a SNALP structure (Figure 4b). Besides, lipidoid molecules can be rapidly synthesized while both their head groups and tails can be easily tailored, which makes it possible to obtain potent lipidoid nanoparticles from a large synthetic library [44, 45]. Since lipidoid nanoparticles are all PEGylated, their gene silencing efficiency will also be discussed in Section 4.2.1.

3.1.4 Multivalent cationic liposomes—Multivalent cationic lipids (MCLs) are developed from monovalent cationic lipids. The head group of an MCL carries more than

one cationic amine group (Figure 2d) and is strongly positively charged in neutral environment. Therefore, MCLs are often formulated in lipoplexes or lipopolyplexes.

The commercial transfection agent Lipofectamine (DOSPA/DOPE 3/1), which contains multivalent cationic lipid DOSPA (Figure 2d), has been widely used in the form of lipoplexes in the *in vitro* transfection of plasmids and siRNAs [22]. Similarly, lipoplexes containing multivalent cationic lipid MVL5 also show high gene transfection efficiency [46]. Interestingly, MLV5 based lipoplexes show much lower toxicity than DOTAP based lipoplexes. This is due to their higher siRNA loading ratio and less cationic lipids involved in the formulations [46].

The reasons why MCLs have high *in vitro* gene silencing efficiency could be: (1) high surface charge leads to high cellular uptake mediated by nonspecific ionic interaction; (2) hexagonal phase in MCL/siRNA complexes lead to high endosomal escape through fusion with endosome membrane.

3.2 Neutral liposomes

Neutral liposomes composed of neutral lipids (Figure 2e) have relatively long blood circulation. Experiments on complement activation and macrophage uptake of differently charged liposomes demonstrated that neutral liposomes did not cause complement activation [34] and had negligible macrophage uptake [33].

Neutral liposomes have the most efficient tumor penetration. Lieleg *et al.* [32] found that neutral liposomes move more quickly than both cationic (>10 mV) and anionic liposomes (< −20 mV) in a tumor-mimic hydrogel. Besides, Nomura *et al.* [47] quantified that neutral liposomes transferred to well-perfused region at a much higher rate (184 times) than cationic liposomes.

The cellular uptake of neutral liposomes is less efficient than that of cationic liposomes [33], and they probably enter cells through nonspecific macropinocytosis, which makes their endosomal escape easier due to the leaky macropinosomes. Besides, neutral liposomes cannot encapsulate siRNA efficiently with passive loading. Hence, special synthesis methods and special structures are necessary to load high amount of siRNAs into neutral liposomes. Therefore, the systemic siRNA delivery of neutral liposomes largely depends on the siRNA loading efficiency and cellular uptake efficiency.

Despite of this, neutral liposomes have satisfying siRNA delivery efficiency *in vivo*. Landen *et al.* lyophilized siRNA/lipid mixture followed by hydration with aqueous buffer to realize high siRNA loading in DOPC liposomes and reduced EphA2 expression *in vivo* with high efficiency [48]. Similarly, Halden *et al.* applied DOPC liposomes to load *FAK* siRNA, which was effective in reducing FAK expression *in vivo* up to 4 days [49].

3.3 Anionic liposomes

Similar to cationic liposomes, anionic liposomes have short circulation time due to complement activation and high macrophage uptake. Also, anionic liposomes have poor tumor penetration, which further retards sufficient tumor accumulation. In addition, anionic

liposomes usually have a low cellular uptake in cancer cells due to repulsive force against anionic membrane. Furthermore, anionic liposomes are not efficient in endosomal escape, either.

What's worse is that simple anionic liposomes have quite low gene loading efficiency, due to the charge repulsion against siRNA. Anionic liposomes can only load sufficient amount of siRNAs with the help of cations or cationic polymers to form stable lipoplexes or lipopolyplexes. Kapoor *et al.* showed that a stable anionic lipoplex of DOPG (Figure 2f) liposome can be synthesized in the presence of calcium ions. This method could knock down 70% of GFP gene at a siRNA dose of only 10 nM *in vitro* [50]. In this lipoplex, calcium ions played an important role in the cellular uptake. At high calcium ion levels, the lipoplexes acted like cationic lipoplexes, entering the cells mostly through proteoglycan-mediated nonspecific ionic interaction; while at low calcium ion levels, the lipoplexes entered cells mostly through macropinocytosis [51], which is similar to neutral liposomes.

3.4 Section summary

Liposomes with different surface charges show their own pros and cons. Monovalent and multivalent liposomes have high cellular uptake and efficient endosomal escape, and therefore are the most robust and most convenient formulations *in vitro*. However, these formulations have short blood circulation and poor tumor penetration, and therefore are not suitable for *in vivo* applications. Neutral liposomes are efficient formulations *in vivo* with satisfactory tumor accumulation and efficient endosomal escape, although their siRNA loading efficiency and cellular uptake efficiency still need to be improved. Anionic formulations are not recommended either *in vivo* or *in vitro*.

4. PEGylation on liposomal siRNA delivery

PEGylation, *i.e.*, surface coating with poly(ethylene glycol) (PEG), is a common method to increase the structural stability of nanoparticles and to extend their circulation time in the blood [52, 53]. PEGylation on liposomes can be efficiently accomplished by either incorporating PEG-conjugated lipids (called PEG-lipids) during the preparation of liposomes (pre-insertion method) [54] or mixing PEG-lipid aqueous dispersions with preformed liposomes afterwards (post-insertion method) [55].

The PEGylation of liposomal carriers can increase the circulation time to meet the requirement for siRNA delivery. However, it would simultaneously reduce cellular uptake [56] and endosomal escape [57], which reduces the final gene silencing efficiency. In this section, we will first summarize the effects of PEGylation on general liposomal carriers and then focus on the effect PEGylation on siRNA delivery.

4.1 The effects of PEGylation on general liposomal carriers

It is known that PEGylation increases the stability and the blood circulation time of liposomes. The efficacy of PEGylation is strongly affected by both the length and the covering density of PEG on liposome surfaces. In general, very short PEG (*e.g.*, PEG1k or smaller) cannot notably reduce the adsorption of proteins and hence cannot extend the circulation time efficiently. To the other extreme, very long PEG (*e.g.*, PEG5k or larger)

would have a strong side effect which severely reduces the cellular uptake and the endosomal escape of liposomes. As a result, medium-sized PEGs (around PEG2k) are the most commonly used ones [58]. For PEG2k, a higher molar ratio to that of total lipids leads to a higher surface coverage [59]:

- PEG <5%: surface coverage <100% by mushroom-like PEG;
- PEG 5~15%: surface coverage ~100% (full coverage) by mushroom-like PEG and brush-like PEG;
- PEG >15%: surface coverage~100% by brush-like PEG. Excess PEG-lipids form micelles.

In general, PEGylation can effectively increase the blood circulation of liposomes by reducing uptake by mononuclear phagocyte system (MPS), which depends on the interaction between liposomes and macrophages. Strong interaction with macrophage leads to short circulation while weak interaction leads to long circulation. Further, the PEGylated-liposome/macrophage interaction depends on two factors: (1) the steric barrier between liposomes and macrophages, (2) protein binding of PEGylated liposomes in the blood. Generally, liposomes with higher density of PEGs have more brush-like conformation and create better steric barriers and therefore have longer circulation time. Therefore, 10% PEG2k of PEGylation on liposomes can greatly enhance the tumor accumulation [60, 61]. While PEGylation is known to reduce macrophage uptake, how protein binding of PEGylated liposomes affect macrophage uptake is controversial. Dos Santos *et al.* incubated PEGylated liposomes with serum for a short period of time (10 min) and concluded that PEGylation can reduce serum protein binding and only 2% of PEG2k is sufficient to inhibit the serum protein binding [62]. However, Johnstone *et al.* incubated PEGylated liposomes (5% PEG2k) with serum for longer time (1 h) and found that PEGylation increased serum protein adsorption and the adsorbed protein changed PEG conformation from mushroom-like to brush-like, reducing macrophage uptake.

Especially, PEGylated liposomes with short PEGs could activate complement system in some circumstances. For instance, PEGylated DPPC liposomes with short PEGs (PEG350) are negatively charged, and hence trigger complement activation and subsequent macrophage uptake [63], leading to shorter blood circulation than unPEGylated DPPC liposomes.

While single dose of PEGylated liposomes is generally believed to have long time circulation, repeated administration of long-circulating PEGylated liposomes leads to accelerated blood clearance (ABC) phenomenon, which severely hinders the therapeutic efficacy. While the exact mechanism is still unknown, the major processes are clear [64]: upon the first injection of PEGylated liposomes, IgM antibodies are produced by activated B cells in the splenic marginal zone; the subsequently injected PEGylated liposomes then interact with the residual IgM antibodies in the serum, activate the complement and are finally taken up by macrophages. The ABC phenomenon is more pronounced at lower first dose (0.001-0.1 μmol lipid/kg) than that at higher dose (5 μmol lipid/kg) [65], empty PEGylated liposomes than some drug-loading [66] or special designed gene-loading liposomes [67, 68], slow vascular infusion other than bolus injection [69], short time interval

between doses (7 days and 21 days) other than long time interval (28 days) [70]. Besides, third (or more) injection of PEGylated lipsomes have less severe ABC phenomenon due to saturation of MPS cells [71]. Therefore, to attenuate ABC phenomenon, bolus injection of higher-dose drug or gene-loaded PEGylated liposomes with longer dosage interval is encouraged.

After the PEGylated liposomes have sufficiently long blood circulation, the next barriers are efficient tumor penetration, cellular uptake, and endosomal escape. A high degree of PEGylation screens the surface charge of liposomes and hence facilitates the tumor penetration, but hinders their uptake in targeted tumor cells at the same time [72]. In other words, for PEGylation there is a trade-off between the blood circulation time and the efficiency of cellular uptake/endosomal escape, which is known as *PEG dilemma*. To circumvent this problem, researchers have introduced cleavable PEG-lipids onto liposomes [73], where PEGs are cleaved from liposome surface after PEGylated liposomes reach tumor tissue. Tumor microenvironment is acidic, reductive and overexpress special enzymes (*e.g.* matrix metalloproteinase). In accordance, different cleavable PEG-lipids are designed to respond to tumor microenvironment, including acidic pH-responsive [74], reductant-responsive [75], or enzyme-responsive PEG-lipids [26] (Figure 3a). Liposomes with all these cleavable PEG-lipids are shown to have higher cellular uptake compared to those with non-cleavable PEG-lipids. Still, their cellular uptake is less than that of non-PEGylated liposomes, which means that the cleaving efficiency needs to be further improved.

4.2 The effect of PEGylation on liposomal carriers for siRNA delivery

4.2.1 PEGylated cationic liposomes

PEGylated monovalent cationic liposomes: For cationic liposome based lipoplexes and lipopolyplexes, PEGylation can effectively reduce the surface charge and increase blood circulation, so that they can be used for *in vivo* siRNA delivery.

PEGylated lipoplexes can be synthesized by both pre-insertion and post-insertion methods. Pre-insertion method is widely used: first PEGylated liposomes (PEG ~1%) are prepared and then lipoplexes are synthesized with the PEGylated liposomes. By this method, Ferring *et al.* reached 80% gene knockdown for CD31 mRNA in lung tumor tissue [54]. However, this method is only suitable for low degree PEGylation. If the ratio of PEG increases to 4-5%, the resulting lipoplexes would have much lower gene silencing efficiency [56, 76]. This decrease is due to much less siRNA loading, since most siRNAs are bound to the exterior of PEGylated liposomes [77]. To obtain high degree PEGylated lipoplexes (PEG ≥ 5%), post-insertion method is a better option. For example, PEGylated DOTAP lipoplexes (10% PEG2k) are very potent, knocking down 50% GFP gene at only 40 nM of anti-GFP siRNA [78].

Lipopolyplexes are generally PEGylated. By the post-insertion method, Li *et al.* incorporated 10% of PEG2k and the formulation showed a high accumulation in the hepatic tumor [79]. DOTAP-based lipopolypolexes with about 10% PEG2k realized high gene knockdown efficiency (50% knockdown of VEGF and TGF-β gene at siRNA doses of 0.6 mg/kg mouse [80], 90% knockdown of anti-CD47 gene at siRNA doses of 12 μg/mouse

[81]). This potency of high degree PEGylated lipopolyplexes can possibly be attributed to *the proton sponge effect* by those polymers in polyplexes during the endosomal escape. In addition, it is of note that in these lipopolyplexes, anionic polymers hyaluronic acid (HA) are incorporated to enhance the stability of polymer/siRNA polyplexes[11, 81].

MCNPs synthesized from cationic liposomes can accommodate even high density of PEGyation without disrupting the bilayer structure, since solid internal NPs support the outer bilayers. For instance, DOTAP-based MCNPs can have 20% PEG2k (Figure 4a) and can silence 60% of luciferase at siRNA dose of 1.2 mg/kg *in vivo* (Figure 4b) [28].

PEGylated pH-sensitive cationic liposomes: The pH-sensitive liposomes are generally PEGylated for *in vivo* application. A commonly used pH-sensitive lipid is DLinDMA (pKa = 6.8). Semple *et al.* [23] synthesized a series of PEGylated pH-sensitive cationic liposomes (SNALP structure with 10% PEG2k) and those containing DLin-KC2-DMA lipid (pKa = 6.7) exhibit *in vivo* activity at low siRNA doses (0.01 mg/kg in rodents and 0.1 mg/kg in nonhuman primates) (Figure 5a). The pH-sensitive cationic liposomes with SNALP structure have high gene silencing efficiency *in vivo*. There are mainly three reasons. First, the encapsulation efficiency of siRNA is usually very high. The high degree PEGylation will not affect the increased membrane permeability by ethanol during the synthesis, which allows the penetration of siRNA into the interior of liposomes. Second, they have neutral surface charge and hence have relatively long circulation time and efficient tumor penetration, which guarantees enough tumor accumulation. Third, they have efficient cellular uptake through clathrin-mediated endocytosis (CME) as well as macropinocytosis [82] and have efficient endosomal escape due to the formation of ionic pairs with endosomal membrane. However, real-time intracellular trafficking shows that only 1-2% of cationic liposomes enter cytosol from endosomes [82], which means that the gene silencing efficiency could be further enhanced after improving the endosomal escape process.

The pH-sensitive lipids can also form lipopolyplexes in acidic conditions (pH = 4.5). Sato *et al.* [83] synthesized PEGylated pH-sensitive cationic liposomes (lipopolyplex structure with 3% PEG2k) containing YSK05 lipid (pKa = 6.5) and found that they are very efficient: the IC_{50} value of gene silencing is less than one-fourth of that of DOTAP formulations.

PEGylated lipidoid nanoparticles: PEGylated lipidoid 98N12-5 (10% PEG2k) [44] can effectively knock down *PCSK9* genes [84] and even knock down multiple genes simultaneously in multiple species [85]. PEGylated lipidoid C12-200 (1.5% PEG2k) knocks down multiple genes at lower doses (0.2 mg/kg per siRNA) [86]. Moreover, some low molecular weight polymeric lipidoid lipids have better performances. PEGylated polymeric lipidoid nanoparticles containing 7C1 (16% or 20% PEG) (Figure 5b) can silence multiple target genes for a long time (Figure 5c) [87]. Similar to pH-sensitive lipids, the pKa of lipidoids also plays an important role in gene silencing effect. Whitehead *et al.* compared PEGylated lipidoid nanoparticles (1.5% PEG) from 1400 types of lipidoids and concluded that the threshold pKa of effective lipidoids is 5.5 [45], *i.e.* lipidoids have a pKa less than 5.5 would be ineffective.

The reasons why PEGylated lipidoid nanoparticles have high gene silencing efficiency are similar to pH-sensitive liposomes except that the cellular uptake mechanisms are different. Lipidoid nanoparticles enter cells mainly through macropinocytosis [88], which should have high endosomal escape. But cell trafficking studies show that about 70% of lipidoid nanoparticles undergo exocytosis from late endosomes [88]. Therefore, enhancing the cytosol delivery efficiency would further enhance gene silencing efficiency.

PEGylated multivalent cationic liposomes: PEGylation are also introduced in multivalent cationic liposomes. Lipoplexes containing multivalent cationic lipid Atufect01 (Figure 2d) with low-degree PEGylation (1-2% PEG2k) are shown to efficiently silence CD31 and Tie2 mRNA of the vascular endothelium at an siRNA dose of 1.88 mg/kg daily *in vivo* (Figure 6) [76], while those with high-degree PEGylation (5% PEG2k) have no gene silencing effect. Lipopolyplexes containing multivalent cationic lipid DSGLA (Figure 5a) with 10% PEG show negligible gene silencing effect *in vitro* [25].

PEGylation severely retards cellular uptake in the case of multivalent liposomes, and only those with 1-2% PEG are plausible for siRNA delivery. It seems that the cellular uptake of multivalent cationic liposomes depends on the interaction between multivalent cationic lipids and cell membrane and high-degree PEGylation will cover the lipids and hence block the cellular uptake.

4.2.2 PEGylated neutral liposomes—The aim of PEGylation is mainly to increase tumor accumulation by increasing blood circulation and tumor penetration, but the neutral liposomes without PEGylation already have high tumor accumulation and additional PEGylation will reduce cellular uptake of neutral liposomes on the contrary. Therefore, it is unnecessary to modify neutral liposomes with PEGs.

4.2.3 PEGylated anionic liposomes—As mentioned before, anionic liposomes are not proper for *in vivo* studies due to their short circulation time, poor tumor penetration and poor cellular uptake. Since PEGylation could increase the circulation time, enhance tumor penetration and increase cellular uptake [33] by screening the surface negative charge, PEGylated anionic liposomes would act similarly to unPEGylated neutral liposomes. However, they have more tedious synthesis procedures and lower siRNA loading efficiency. Therefore, PEGylated anionic liposomes are not encouraged for *in vivo* applications.

4.2.4 PEGylated liposomes with cleavable PEGs—As previously discussed, liposomal carriers modified by cleavable PEG would perform better than those modified by uncleavable ones. Hatakeyama *et al.* [26] synthesized lipopolyplexes with MMP-cleavable PEG, named PPD/PEG5k-MEND, which not only had a higher tumor accumulation (Figure 3b), but also showed notably better gene silencing efficiency *in vivo* owing to those cleavable PEGs (Figure 3c).

However, liposomes with cleavable PEG are still less efficient than bare liposomes, since cleavable PEGs cannot be totally cleaved from liposome surface. Therefore, “super-cleavable” PEGs with faster and stronger response to tumor environment need to be developed.

5. Surface targeting ligands on siRNA delivery

On tumor cell surface, a lot of receptors are overexpressed and corresponding surface ligands can specifically bind to them. Hence, surface modification on liposomes by targeting ligands can enhance cellular uptake in tumor cells by receptor-mediated endocytosis and further increase the gene silencing efficiency [89, 90]. Besides, some peptides like GALA [91] can disrupt endosomes after endocytosis and are also conjugated to liposome surface to increase final gene silencing efficiency. Common targeting ligands include monoclonal antibody (mAb) fragments (*e.g.* fragment antigen-binding (Fab) and single-chain variable fragment (scFv)), proteins, peptides, vitamins, carbohydrates, glycoproteins, and other small molecules [89].

While surface modification increases cellular uptake of liposomes, it also increases the complexity during blood circulation [92] and tumor penetration. One way out is to cover the targeting ligands with cleavable PEGs, which will detach from liposome surface in the tumor microenvironment [93-95]. However, the cleavable PEGs are still far from mature at present and targeting ligands are exposed on liposome surface [91, 96] in most cases.

5.1 Surface ligand targeted cationic liposomes

As mentioned before, PEGylated cationic lipoplexes, lipopolyplexes, SNALPs and MCNPs are promising in *in vivo* siRNA delivery. Further modification with targeting ligands can increase gene silence efficiency.

Targeted lipoplexes—For most targeted lipoplexes, the targeting ligands are modified at the end of modified PEGs to increase cellular uptake while the amount of targeting ligands varies case by case. For example, phage fusion coat protein-modified cationic lipoplexes (2% PEG2k molar ratio, 0.5% protein w/w) targeting MCF-7 cell have two-fold higher gene silencing efficiency than that of non-targeting ones in *in vitro* cancer targeting [97]. Anti-E-selectin-conjugated lipoplexes (4% PEG2k and 1% PEG2k-antibody) targeting activated endothelial cells have about 50-fold higher cellular uptake than non-targeting liposomes in the presence of TNF-α cytokine and can knock down 60% of gene in H5V cells [98]. RGD-modified lipoplexes (10% cRGD-PEG5k) targeting integrin $\alpha_v\beta_3$ overexpressed on cancer cell surface can double the gene silencing efficiency compared with non-targeting ones in the *in vivo* lung cancer targeting [99].

In some cases, high amount of targeting ligands eliminate the effect of incorporated PEGs in lipoplexes. For example, galactose-modified lipoplexes (62.5% lipid-galactose, no PEG) targeting asialoglycoprotein receptor on hepatic cancer cells show a fast accumulation in liver tissues, which is three times than the non-modified ones [100].

Targeted lipopolyplexes—For targeted lipopolyplexes, the amount of targeting ligands and PEGs also varies case from case. For example, GALA (endosomalytic)-modified lipopolyplexes (1% PEG2k, 2% cholesterol-GALA), carrying anti-*ATCB* or anti-*luciferase* siRNA, resulted in four times gene silencing efficiency of non-targeted ones *in vitro* and two times in HT1080 tumor-bearing mice [91]. GC4 scFv-conjugated lipopolyplexes (8% scFv-PEG2k) targeting B16F10 cells showed 2-3 fold higher gene silencing efficiency compared

with non-targeting lipopolyplexes in lung lobe (Figure 7a) [101]. Galactose-modified lipopolyplexes (10% galactose-PEG2k) have about 3-fold more gene silencing efficiency than that of non-modified ones *in vivo* [102, 103]. AA-targeted lipopolyplexes (10% PEG and 10% AA-PEG) targeting sigma-receptors overexpressed on cancer cell surface knocked down 90% CD47 in melanoma at a dose of 0.6 mg/kg [81]. Another AA-targeted lipopolyplex formula (20% AA-PEG) also had a higher cellular uptake and significantly higher gene silencing efficiency than non-targeted formulations both *in vitro* and *in vivo* [25].

Other targeted cationic liposomal formulations—Other targeted cationic liposomal formulations (SNALPs and MCNPs) are less studied. For instance, folate-conjugated SNALPs (2.5% PEG, 2.5% folate-PEG) targeting folate receptors on cancer cells can knock down 40% of targeted MYCN gene *in vivo* [104]. AA-modified MCNPs (11% PEG, 11% AA-PEG) have three times gene silencing efficiency compared with non-modified LCPs [105].

Finally, it is interesting to note that the degree of PEGylation can be as high as 20% in the presence of surface ligands, which means that surface ligands can partially alleviate the side effect of PEGylation on cellular uptake.

5.2 Surface ligand targeted neutral liposomes

Neutral formulations are often considered less efficient than cationic formulations in *in vitro* studies. But targeted neutral formulations can greatly increase the cellular uptake and can have even higher gene silencing efficiency than lipoplexes in some cases. For instance, cRGD-modified neutral MCNPs (3% cRGD-PEG) targeting integrin $\alpha_v\beta_3$ have higher gene silencing efficiency than *Lipofectamine* 2000 but with lower toxicity *in vitro* (Figure 7b) [30]. Similarly, neutral MCNPs modified with SP94 peptide targeting HCC and peptide H5WYG targeting endosomes can totally silence multiple genes simultaneously *in vitro* [29].

Up to now, there are many types of liposomal siRNA carriers which incorporate different targeting ligands. Such surface modification method is convenient, and the key is the design of targeting ligand molecules. By properly selecting the ligand, the gene silencing efficiency can be substantially increased over that of non-targeting ones. As a result, targeting ligand modification of liposome carriers is a very promising approach for future studies.

6. Conclusions and perspectives

In this paper, we gave an overview of recent developments in the liposome-based systemic siRNA delivery. The main types of liposome carriers for siRNA delivery can be categorized into four types: lipoplexes, SNALPs, lipopolyplexes, and membrane/core nanoparticles. Surface properties of liposome carriers, including surface charges, PEGylation, and targeting ligand modification, significantly affect the gene delivery efficiency.

In general, common cationic liposomes with lipoplex structures, composed of monovalent or multivalent cationic lipids, are the most robust and widely used formulations, which show a

good performance *in vitro*. The pH-sensitive liposomes (SNALPs), which incorporate pH-sensitive lipids and lipidoids, are more potent in the *in vivo* siRNA delivery due to their long circulation time and efficient tumor penetration, thanks to their neutral charged surfaces. Their potency can be further improved by enhancing endosomal escape. Neutral liposomes have good performance both *in vitro* and *in vivo*. Anionic liposomes are not encouraged in *in vitro/in vivo* cases.

PEGylation prolongs the circulation and tumor penetration of both cationic and anionic liposomes. Neutral liposomes generally do not need PEGylation to increase circulation. Due to the poor cellular uptake, PEGylated anionic liposomes are not encouraged. Therefore, PEGylation is mostly applied to cationic liposomes. With PEGylation, cationic liposomes can be used in *in vivo* siRNA delivery. However, repeated injection of PEGylated liposomes cause accelerated blood clearance. To attenuate ABC phenomenon, bolus injection of higher-dose drug or gene-loaded PEGylated liposomes with longer dosage interval is encouraged. Besides, PEGylation reduces cellular uptake/endosomal escape of cationic liposomes and there exists a trade-off between the circulation time and the cellular uptake/endosomal escape. To solve the problem, cleavable PEGylated liposomes under tumor microenvironment are developed and they can reduce the side effects without affecting the PEG protection, which leads to an improved performance compared with classical non-detachable PEGs.

Finally, incorporation of proper targeting ligands into the formulations would significantly increase the efficiency of the siRNA delivery.

With certain successes in the liposomal siRNA delivery, there are still many problems remain to be answered. Figure 8 summarizes the problems and potential solutions. The first problem is the lack of systematic studies of the side effect of surface ligands during the blood circulation and tumor penetration. In some cases, surface ligands would cause immune response and poor tumor penetration. The relation between the chemical structures of surface ligands and the immune response/tumor penetration is virtually unexplored. The second problem relates to PEGylation. In theory, cleavable PEG is an efficient solution for the PEG dilemma and the ABC phenomenon induced by repeated PEGylated formulations. While cleavable PEGylated liposomes work better than uncleavable PEGylated ones in both *in vitro* and *in vivo* studies, the cleavage efficiency is suboptimal, since *in vitro* studies show that current cleavable PEGylated liposomes are still less efficient than unPEGylated ones. Researches on super-cleavable PEGs are strongly needed. The third problem is that the detailed cellular uptake mechanism of cationic formulations (lipoplexes and lipopolyplexes) is still not fully elucidated: is it just non-specific ionic interaction-mediated endocytosis, or is it regulated by some underlying factors? Moreover, after the uptake stage, how much of the uptaken siRNA has successfully entered cytosol from endosomes, and where are the siRNA molecules that failed in endosomal escape? The study of the detailed mechanism depends on advanced cell trafficking techniques, which may facilitate further optimization of cationic formulations. Furthermore, the development of molecular imaging techniques may provide effective measures on the systemic siRNA delivery process.

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Appendix

Abbreviations

AA	anisamide
CaP	calcium phosphate
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt)
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOSPA	2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DC-chol	3-β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride
DDAB	dimethyldioctadecylammonium (Bromide Salt)
DLin-DMA	1,2-dilinoleyloxy-3-dimethylaminopropane
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt)
DSGLA	(N,N-distearyl-N-methyl-N-2[N′-(N2-guanidino-L-lysinyl)] aminoethyl ammonium chloride)
EGFR	epidermal growth factor receptor
Fab	fragment antigen-binding
mAb	monoclonal antibody
MEND	multifunctional envelope-type nano device
MNCP	membrane/core nanoparticle
MVL	multivalent lipid
RISC	RNA-induced silence complex
scFv	single-chain variable fragment
siRNA	small interfering RNA
SNALP	stable nucleic-acid-lipid particle
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

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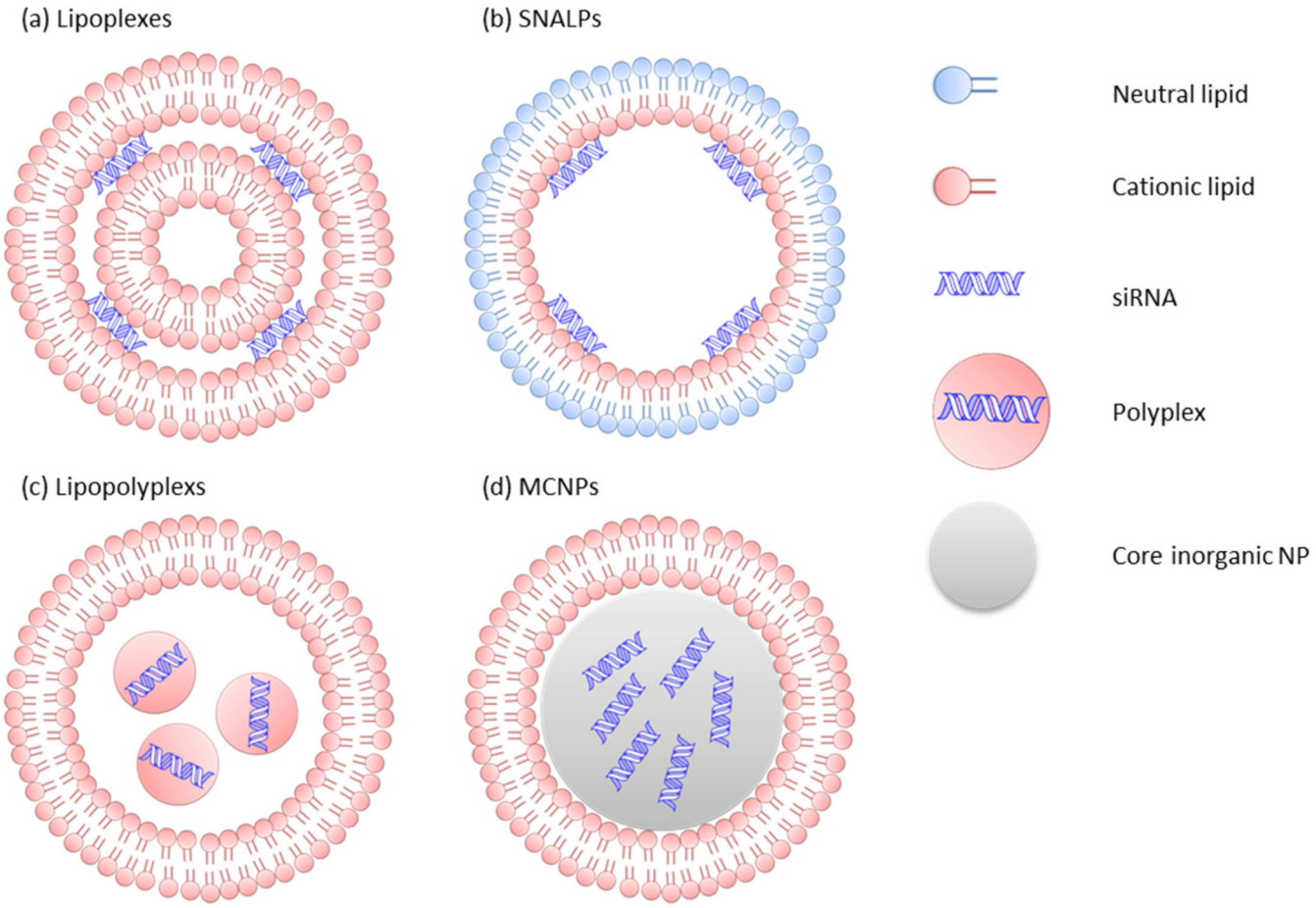

Figure 1.

Four liposomal structures for siRNA delivery: a) lipoplexes; b) SNALPs; c) lipopolyplexes; d) MCNPs.

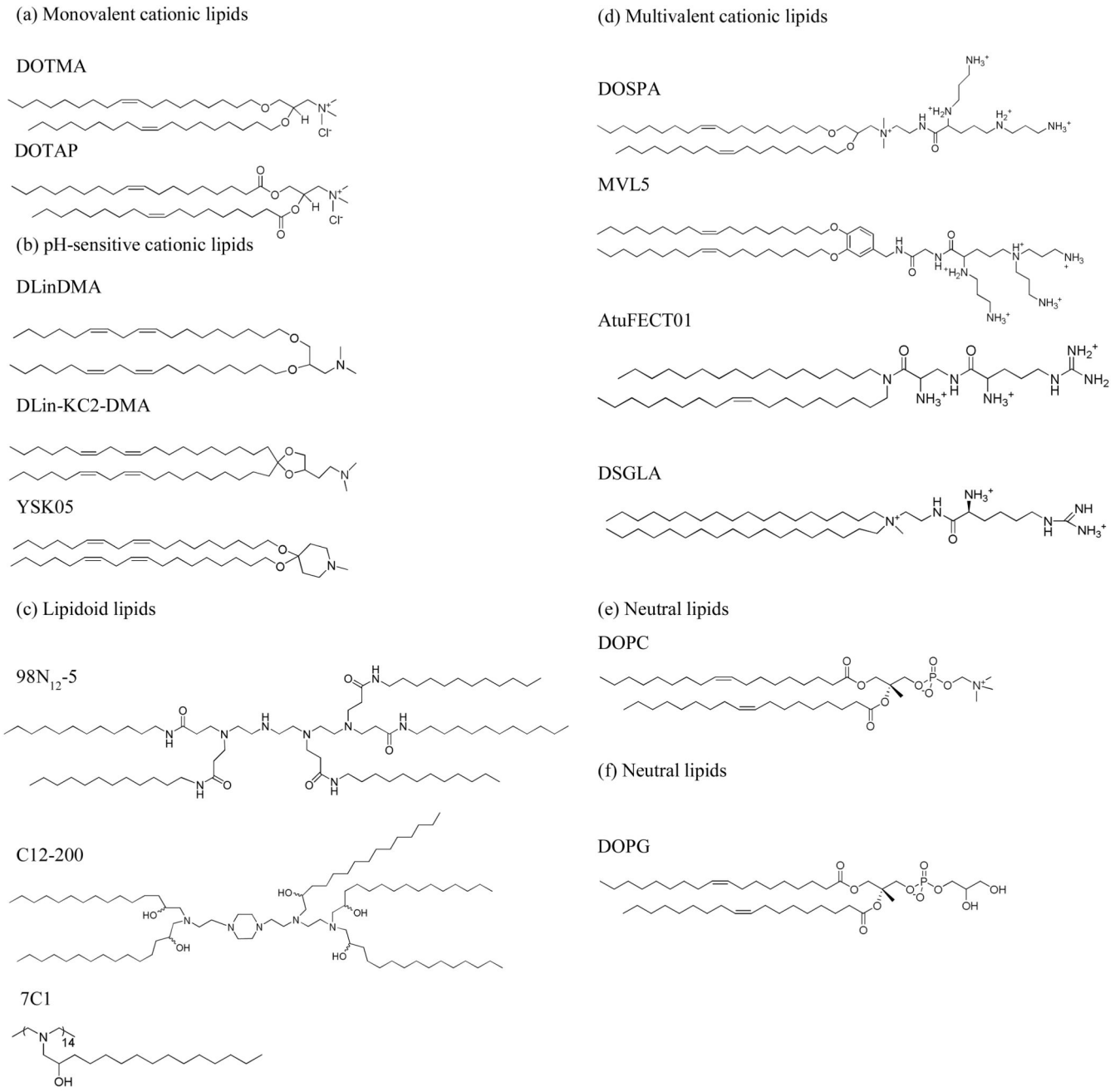

Figure 2.

Chemical structures of differently charged lipids: (a) monovalent cationic lipids; (b) pH-sensitive cationic lipids; (c) lipidoid lipids; (d) multivalent cationic lipids; (e) neutral lipids; (f) anionic lipids.

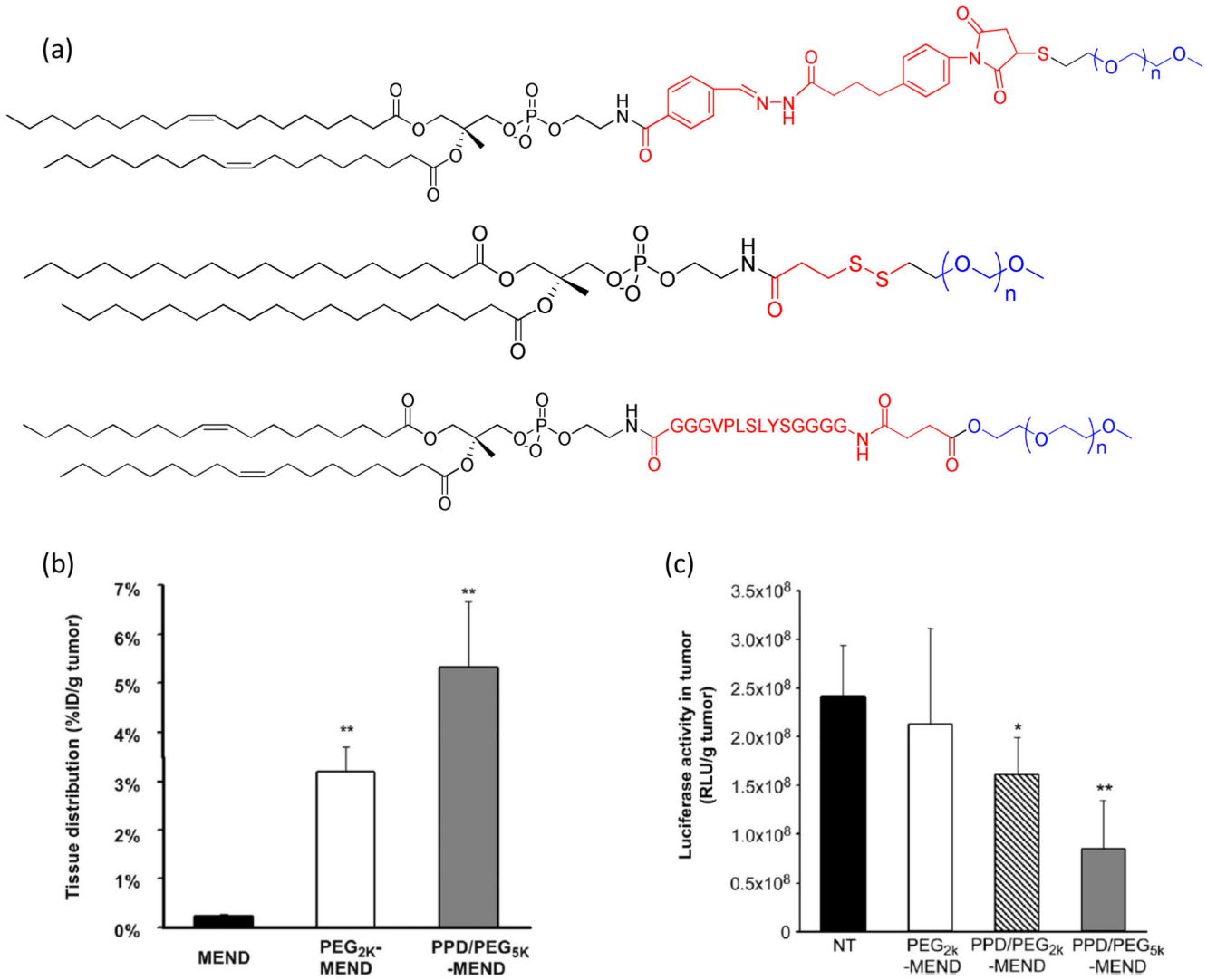

Figure 3.

(a) Chemical structures of cleavable PEG-lipids: pH-sensitive PEG2k-HZ-PE [106]; reductive environment sensitive DSPE-S-S-PEG5k [75]; MMP-sensitive PPD (PEG2k-peptide-lipid) [26]. Lipid, linker, and PEG segments are in black, red and blue, respectively. (b) Tumor accumulation at 24 h after administration of different MENDs: MEND (0% PEG), PEG2k-MEND (15% PEG2k) and PPD/PEG5k-MEND (11.25% PPD, 3.75% PEG2k) [26]. (c) The luciferase activity in tumor tissue at 24 h after the systemic administration of PEG2k-MEND (15% PEG2k), PEG2k-DSPE PPD/PEG2k-MEND (7.5% PPD, 7.5% PEG2k), and PPD/PEG5k-MEND (11.25% PPD, 3.75% PEG2k) [26].

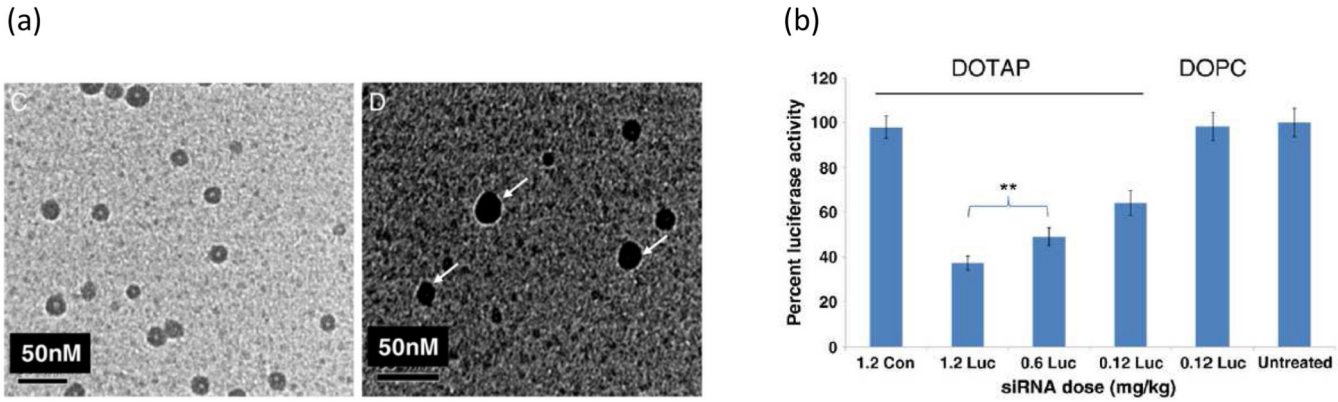

Figure 4.

(a) TEM images of DOTAP-based MNCPs without (left) or with (right) negative staining. Adapted from [28]. (b) The *in vivo* silencing effect of luciferase siRNA delivered by DOTAP-based and DOPC-based MNCPs at different doses. Adapted from [28].

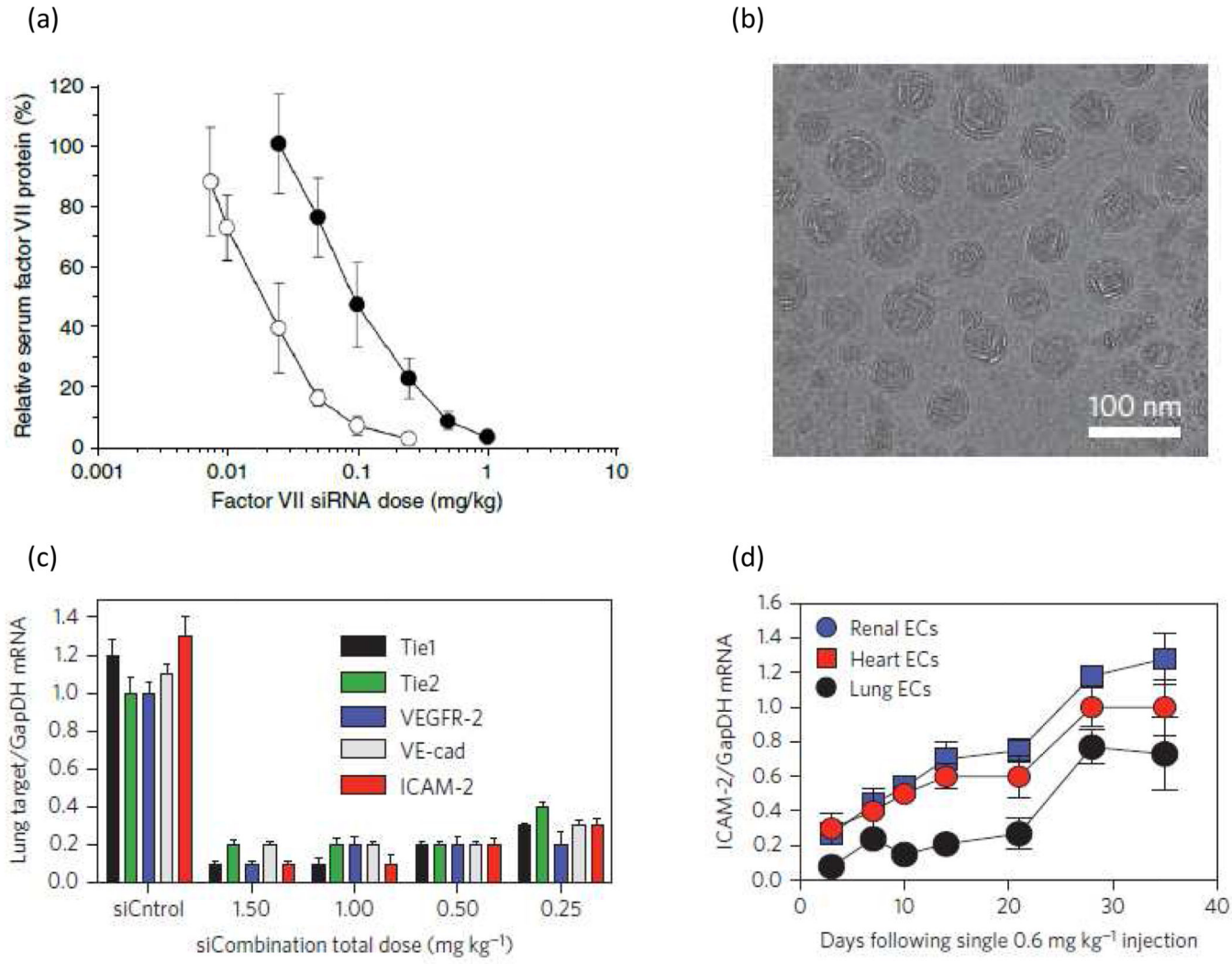

Figure 5.

(a) The relative serum factor VII protein after the administration of optimized (circle) and unoptimized (dot) DLin-KC2-DMA formulations in mice. Adapted from[23]. (b) TEM images of lipidoid 7C1 based lipidoid nanoparticles. Adapted from [87]. (c) Target/GAPDH mRNA ratios (normalized to PBS-treated mice) following the injection of 7C1 formulated with control siRNA and five siRNAs targeting ICAM-2, Tie2, VE-cadherin, VEGFR2 and Tie1, respectively. Adapted from [87]. (d) ICAM-2/GAPDH mRNA levels as a function of time following a 0.6 mg/kg injection of ICAM siRNA. Adapted from [87].

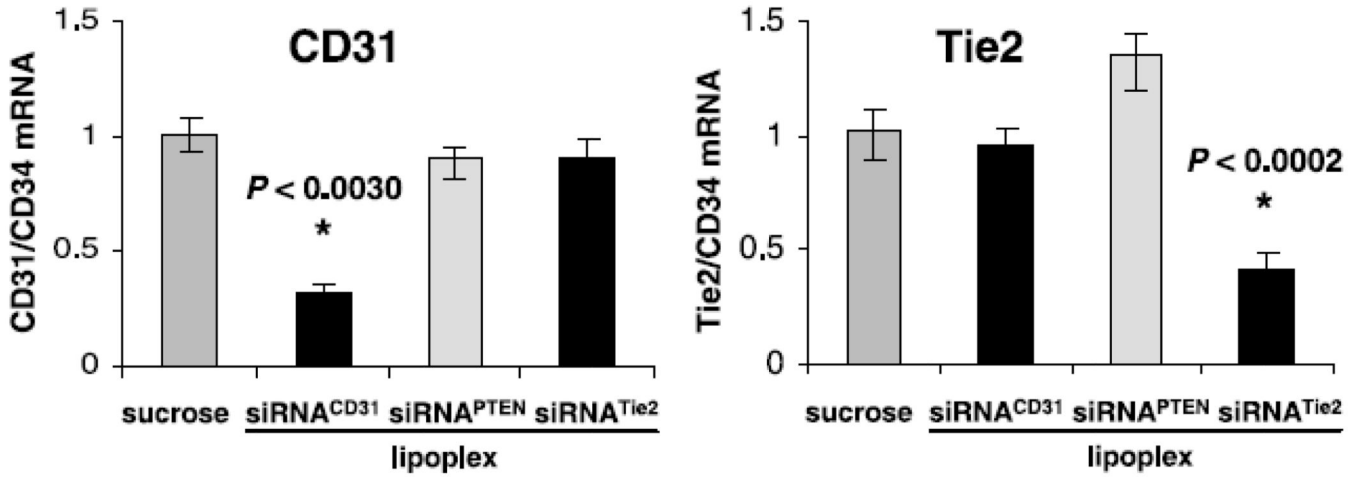

Figure 6.

Knockdown of mRNA levels for Tie2 (right diagram) or CD31 (left diagram) in the lung with four consecutive daily i.v. injections (daily dose: 1.88 mg/kg siRNA) with target-specific siRNA-lipoplexes [76].

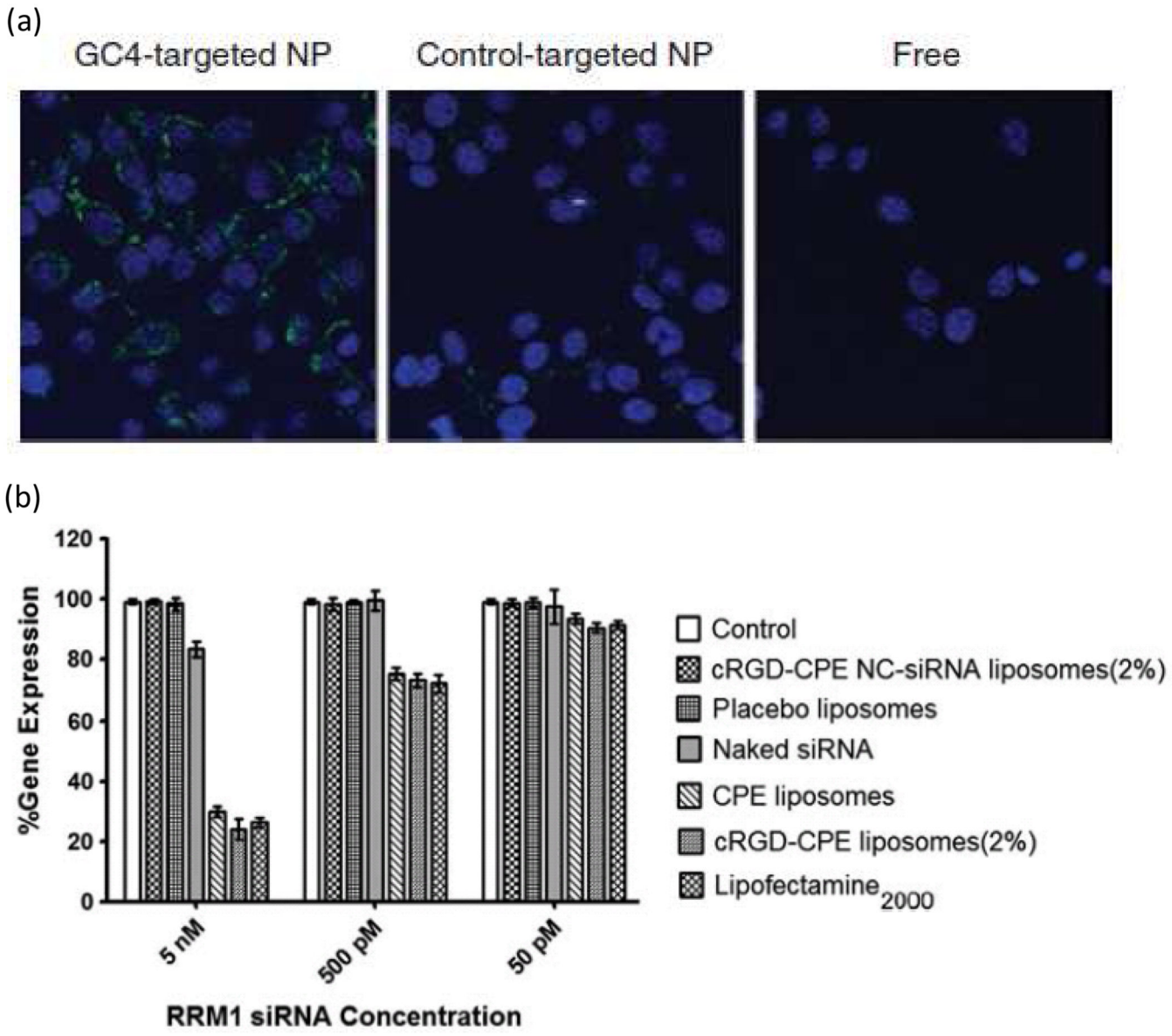

Figure 7.

(a) Fluorescence photographs of B16F10 cells after the treatment with free siRNA or siRNA formulated in the GC4-targeted nanoparticles for 1 h. Fluorescence signal of FITC-labeled siRNA in B16F10 cells was observed by the confocal microscopy [101]. (b) Silencing effect of RRMI siRNA in A549 cells by neutral membrane/core nanoparticles (cRGD-CPE liposomes) with Lipofectamine 2000 as positive control. Adapted from ref [30].

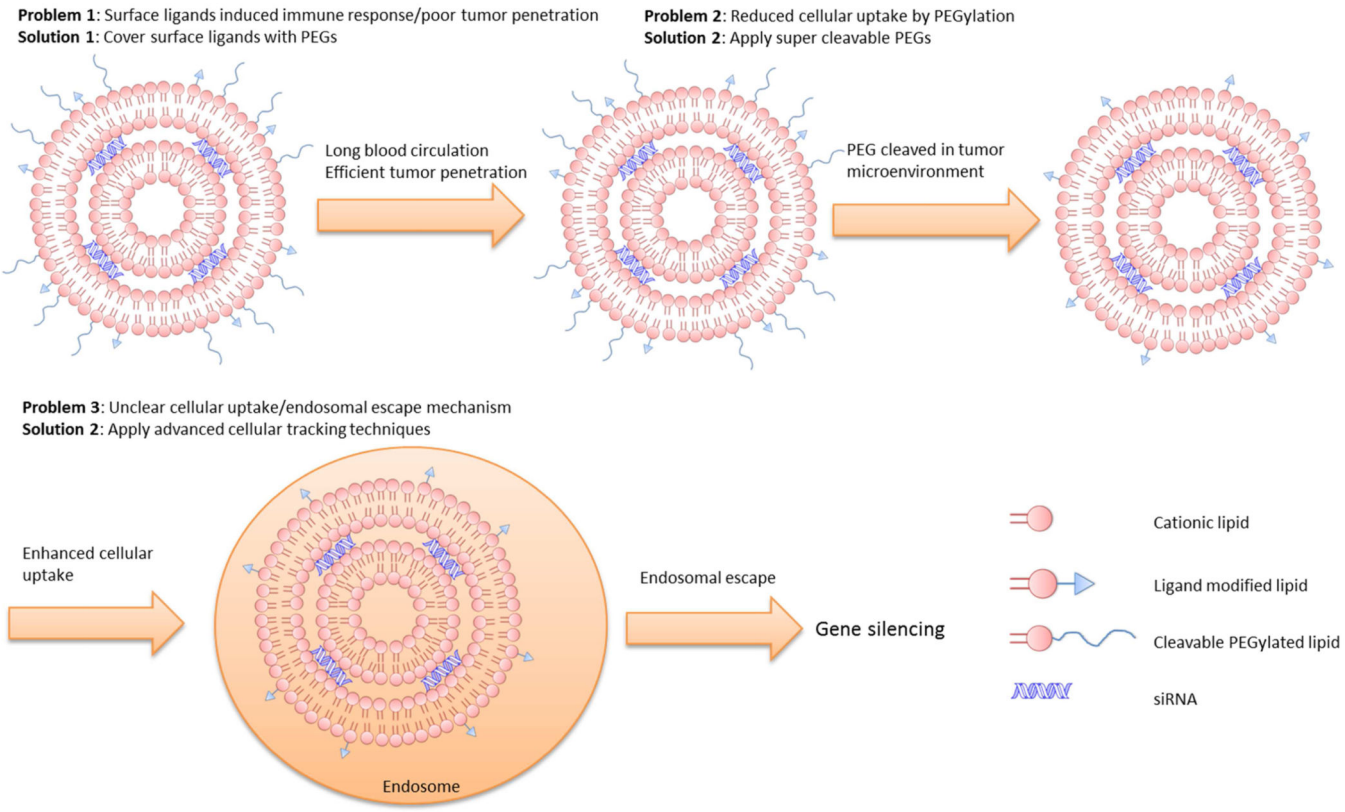

Figure 8.

A scheme systemic delivery of liposomal siRNA formula that can partly solve current delivery problems: (1) inhibition of surface ligand induced immune response/poor tumor penetration by covering surface ligands with PEGs; (2) increase of PEGylation caused less cellular uptake by applying cleavable PEGs; (3) investigation of cellular uptake/endosomal escape mechanism by applying advanced cellular trafficking techniques.