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## Diversity of PEGylation methods of liposomes and their influence on RNA delivery

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Gene therapy is a promising approach for personalized medicine, but its application in humans requires development of efficient and safe vehicles. PEGylated liposomes are some of the most suitable delivery systems for nucleic acids because of their stability under physiological conditions and prolonged circulation time, compared to conventional and other types of “stealth” liposomes. *In vitro/in vivo* activity of PEGylated liposomes is highly dependent on PEG motif abundance. The process of “stealth” coverage formation is a very important parameter for efficient transfection assays and further fate determination of the PEG layer after tissue penetration. In this review, we discuss the latest methods of PEGylated liposome preparation.

### Introduction

RNA-interference (RNAi) is a biological process related to mRNA expression inhibition by double-stranded RNAs (dsRNAs). In RNAi processing, short dsRNAs dissociate into single-stranded RNAs, which conjoin with RNA-induced silencing complex (RISC) proteins. Thereafter, the RISC binds complementarily to mRNA and cuts polynucleotide chains, so that the target mRNA expression is inhibited.<sup>1</sup> There are two main types of short dsRNAs used for RNAi: endogenous microRNA (miRNA) and artificially synthesized small interfering RNA (siRNA).

A number of RNAi therapeutic candidates have emerged with profiles suggesting that they could become drugs of significant medical importance for diseases like transthyretin amyloidosis, hepatitis B virus infection, solid cancers, and hemophilia.<sup>2,3</sup>

Despite this robust progress, the current state of RNAi therapeutics development has a particular focus on RNAi delivery and key challenges should be faced to expand therapeutic opportunities.<sup>4</sup>

Due to the short life span of siRNA in blood serum (about 15 min) and its total negative charge, which hinder siRNA pervasion through the negatively-charged cell membrane, siRNA needs to form nano-sized complexes with carriers for facile intracellular delivery.<sup>5</sup> Nanocarriers should exhibit:

- prolonged circulation time;
- effective tissue permeation; and
- a high degree of cellular uptake and successful endosomal escape.

Currently, liposomes are the most investigated siRNA delivery vehicles. Their significant biodegradability and biocompatibility enable their successful entry into clinical investigations. Cationic liposomes are spheroidal nanoparticles with a bilayer structure based on amphiphilic lipids, which resemble the cellular membrane, providing better cellular uptake and fewer side effects, compared to lipid nanoparticles. The latter are spheroidal solid nanoparticles at room and body temperature and loaded with lipophilic compounds, mainly. Thus, cationic liposomes seem to be suitable vectors for siRNA delivery.<sup>6–8</sup>

### “Stealth” liposomes

To obtain “stealth” or sterically stabilized liposomes, modification of the bilayer surface with inert polymers, which control semi-phase processes and prevent interaction of liposomes and blood components, exhibiting the so-called “stealth” properties is used. Improvement of the “stealth” properties of nanoparticles is the key goal of drug delivery, as the efficiency of drug delivery systems is closely related to their circulation time. Prolonged circulation time, low MPS (mononuclear phagocyte system) cellular uptake, low aggregation in blood serum and storage stability are main advantages of “stealth” liposomes.<sup>9</sup> The most successful methods of “stealth”-mask creation were achieved by liposomal surface modification with polyacrylamide, polyvinyl alcohol and polycarbohydrates, along with polyethylene glycol (PEG) and its co-polymers.<sup>10–12</sup> In the very first preparation strategy liposomes mimicked the red blood cell membrane. This tactic allowed them to co-exist with macrophages in the blood stream. For this purpose the liposomal surface was covered with sterically bulky ganglioside GM<sub>1</sub> or phosphatidylinositol (PI), both leading to sufficiently prolonged circulation time.<sup>13,14</sup>

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It was suggested that other flexible hydrophilic polymers can efficiently increase the blood circulation time. In particular, the use of PEG-containing lipids with molar weights of 1900–5000 Da prolonged the circulation time compared to GM<sub>1</sub> and PI.<sup>15–19</sup> The strategy of application of PEG as a covering polymer for ‘stealth’ liposome formulation is known as PEGylation.<sup>20</sup>

## PEGylated liposomes

PEGylation can be carried out in two ways: addition of PEG-lipids to lipid composition before liposome formation (pre-insertion method) or mixing of PEG-lipids and liposomal dispersion (post-insertion method).

Both the length and coverage density of PEG influence the liposomal PEGylation efficiency. Very short PEG molecules cannot prevent protein absorption and increase the blood circulation time, while very long PEG chains lead to significant decline in transfection activity. Usually PEG molecules with medium length are used for liposome modification. The higher the molar PEG-lipid/lipid composition ratio, the higher the coverage density (Fig. 1):

- <5% PEG gives lower than 100% coverage, mushroom-like shape;
- 5–15% PEG gives ~100% coverage, mushroom- or brush-like shape;
- >15% PEG gives ~100% coverage, brush-like shape.

Liposome PEGylation increases the circulation time by inhibiting the MPS uptake,<sup>21,22</sup> *i.e.* preventing liposome–macrophage interaction. PEG-liposomes also tend to accumulate in tumor foci.<sup>15</sup> Interaction between PEG-liposomes and macrophages depends on two factors: the steric barrier between liposomes and macrophages and binding to blood proteins. In general, liposomes with a brush-like coverage have a more stable steric barrier and prolonged circulation time.<sup>23</sup> However, PEGylated liposomes demonstrate remarkably lower cellular uptake and endosomal escape, which reduces the final gene silencing effect.

It is considered that repeated PEG-liposome administration leads to rapid blood clearance, which decreases the therapeutic effect of the drug significantly. After the first administration, activated B-cells produce IgM antibodies. The following administration causes drug–IgM binding, complement system activation and liposome capturing by macrophages.<sup>24</sup> This phenomenon is a very fast immune reaction that occurs in 10 min after first intravenous injection.<sup>25</sup> Moreover, the spleen seems to be the main producer of anti-PEG IgM.<sup>26</sup> It should be noted that secretion of IgM antibodies is dose dependent and that the immune response activation rate is higher when triggered by nucleic acids.<sup>27</sup> Nevertheless, the third and later administrations of PEG-liposomes do not cause such a strong effect due to MPS cell saturation.

### Pre-insertion PEGylation

Traditionally, PEGylated liposomes are obtained by the pre-insertion method. Liposomal particles are formed by thin lipid film hydration with the help of sonication.<sup>28</sup> Cationic, anionic or neutral lipids are mixed with lipids containing PEGs at their head group on the thin film step.<sup>29–31</sup> Then, nucleic acid (NA) is added to form PEGylated lipoplexes (Fig. 2).<sup>32,33</sup> This method is suitable for liposomes with low PEG motif density on the periphery (approximately 1%).<sup>34</sup> The most commonly used compounds for liposomal PEGylation (Fig. 3) are various phosphatidylethanolamines (PE): DSPE-mPEG,<sup>35–37</sup> DPPE-mPEG,<sup>38</sup> and DOPE-mPEG,<sup>39</sup> which differ in: i) hydrophobic domain; ii) PEG chain length and iii) fatty acid type.

It was demonstrated that PEG derivatives with a molecular mass of 2000 Da are the most appropriate for the stabilization of liposomal structures in the bloodstream.<sup>40</sup> *In vivo* experiments proved that liposomes modified with PEG<sub>2000</sub> are four times more effective in tumor tissue targeting than non-modified liposomes.<sup>41</sup> The newest technologies in polymer synthesis (combination of liquid-phase iterative synthesis and selective molecular sieving) give researchers the

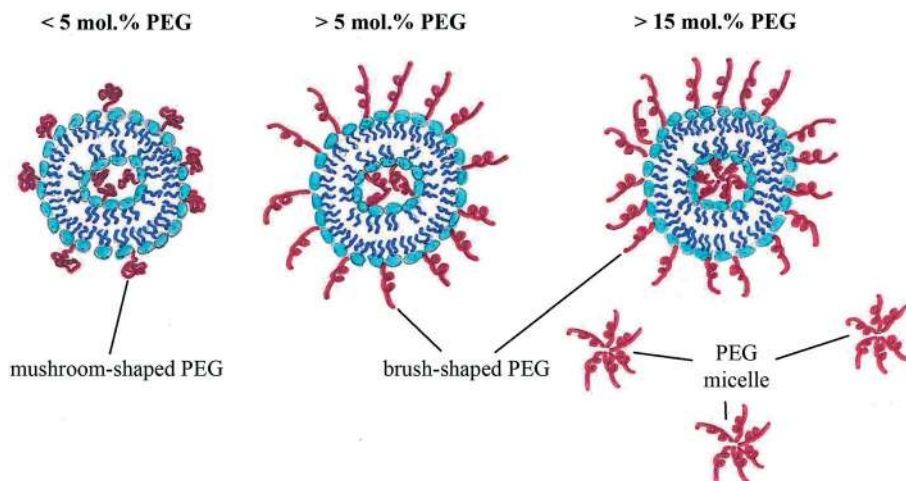


Fig. 1 PEGylation density – coverage type dependency.

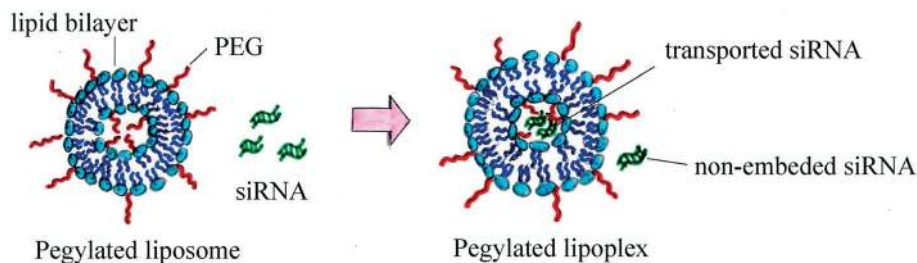


Fig. 2 Scheme of PEGylated lipoplex preparation by the pre-insertion method.

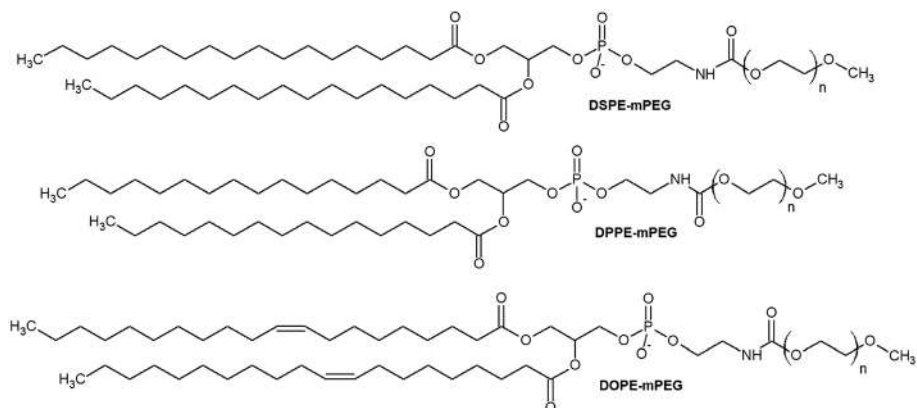


Fig. 3 mPEG-PE lipids: *N*-(methylpolyoxyethylene oxycarbonyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-mPEG), *N*-(methylpolyoxyethylene oxycarbonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE-mPEG), and *N*-(methylpolyoxyethylene oxycarbonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethylene glycol) (DOPE-mPEG). *N* – number of oxyethylene  $-\text{CH}_2\text{CH}_2\text{O}-$  units.

opportunity to use defined-sequence PEGs.<sup>42</sup> PEGs with an exactly defined length and number of oxyethylene units have great biocompatibility and purity.

The transfection efficiency and cellular uptake of PEGylated liposomes are affected not only by the PEG chain length but also by the lipid hydrophobic domain nature.<sup>43,44</sup> Short aliphatic derivatives easily diffuse from the liposomal surface which results in a decrease of the prolongation effect,<sup>45</sup> while asymmetry of the lipophilic part promotes fusion between lipoplexes and the cell membrane.<sup>46</sup> Steroids and lipopeptides can also be employed as bases of PEGylated lipids (Fig. 4). For example, PEG-lipopeptides like mPEG2k-Glu(C<sub>16</sub>)<sub>2</sub> have the right balance between low cytotoxicity and good pDNA/siRNA transfection activity.<sup>47,48</sup> It was noticed that cholesterol-based PEGylated lipoplexes tend to accumulate mainly in hepatic tumors<sup>49</sup> and may have great potential in gene therapy of liver diseases. Liposomes modified with mPEG-Chol (Fig. 4) also demonstrate high dispersion stability compared to PEGylated liposomes which contain PEG-PE lipids.<sup>46</sup>

To date several drugs based on PEGylated liposomes have been approved by the FDA and already entered the market. They encapsulate and transport doxorubicin,<sup>50,51</sup> vincristine<sup>52</sup> and daunorubicin.<sup>53</sup>

When PEG-containing lipids are added during thin lipid film formation, PEG chains become present on both the external and internal membrane parts of the lipid bilayer. PEG motifs present inside the liposome do not form a protective

coverage, but hinder siRNA loading into the liposome (Fig. 2). The post-insertion method allows modification of only the outer liposomal bilayer surface.

### Post-insertion PEGylation

The post-insertion method (Fig. 5) is more effective in reaching a high level of PEGylation (more than 5% PEG).<sup>54</sup> Here, the reaction between a PEG derivative and special hydrophobic anchor or matrix lipids in the outer surface of the liposomal membrane occurs, resulting in the presence of PEGylated conjugates only on the liposomal external lipid bilayer surface.<sup>55</sup> This method is applied for biomolecule delivery and liposome-based drug design<sup>38,56</sup> due to a suitable ligand arrangement on the outer liposomal surface and a high

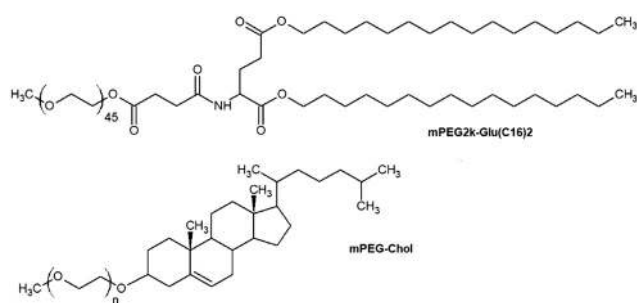


Fig. 4 Non-PE PEGylated lipids: mPEG2k-Glu(C<sub>16</sub>)<sub>2</sub> and mPEG-Chol. *N* – number of oxyethylene  $-\text{CH}_2\text{CH}_2\text{O}-$  units.

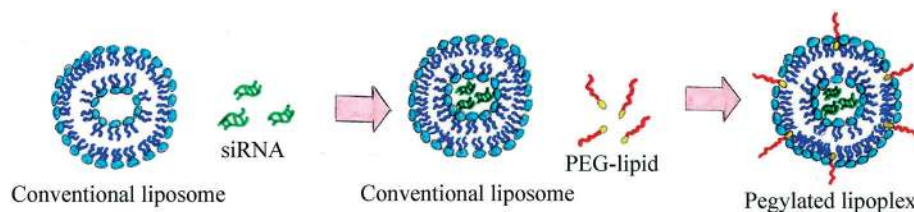


Fig. 5 Scheme of PEGylated lipoplex preparation by the post-insertion method.

level of embedding under optimal conditions leading to a decrease in non-specific interactions between ligands and liposomes.

Usually, post-insertion is completed after the drug loading step and performed by slow addition of the PEG-lipid solution or PEG micelles to the liposomal dispersion followed by removal of excess PEG derivatives *via* centrifugation and filtration. Liposomes that are prepared by the post-insertion technique are commonly as stable as the pre-PEGylated ones, but do not have their limitations like low encapsulation rates and weak transfection efficiency.<sup>56,57</sup>

This method does not influence the conventional drug loading technique.<sup>57,58</sup> Data from several studies have shown that post-insertion of mPEG-HDAS (Fig. 6) inhibits the complement system activation<sup>59</sup> and this lipid penetrates the bilayer significantly higher than mPEG-PEs. PEGylated ceramides (Fig. 6) are also used for post-insertion, showing better transfection efficiency of liposome/siRNA complexes compared to liposomes obtained by the pre-insertion method due to hexagonal phase formation.<sup>45,60</sup> The post-insertion technique is a promising approach for the gene delivery of liposomal systems to cancer cells.<sup>61,62</sup>

The reaction between a PEG derivative with a terminal azide group and a hydrophobic alkyne-ended anchor built into the lipid bilayer by a “click” mechanism is an example of the PEGylated liposome formation by the post-injection method by means of chemical bonding.<sup>63,64</sup> This reaction of

1,3-dipolar cycloaddition proceeds practically with a quantitative yield and only on the outer surface of the lipid bilayer.<sup>61</sup> Sebyakin *et al.* showed that conjugation of modified PEGs to an azide-containing lipopeptide immersed in the liposomal bilayer leads to mPEG2k-tz-Glu(C<sub>16</sub>)<sub>2</sub> (Fig. 6) formation. It can proceed after liposome formation and loading stages.<sup>65</sup> However, this kind of post-insertion of PEGs is poorly covered in the literature, and still the “click”-reaction bonding of various ligands to prepared liposomes is highly applied. In this approach PEG motifs are used as spacers and show good ability for modifications with maleimide, thiol and hydrazone terminal groups.<sup>37,66,67</sup> These data promise significant success in the post-insertion method development.

Despite the great transcendence of PEGylated lipoplexes, the use of classical PEG-lipids (both by pre-insertion and post-insertion) brings nanomedicine to the “PEG dilemma” – the balance between prolongation of the blood circulation time and cellular uptake/endosomal escape reduction.<sup>68,69</sup> It is known that cationic lipids have two conformations in aqueous solution, they are lamellar and hexagonal phases.<sup>70</sup> Liposomes that can rearrange their bilayer into a hexagonal phase after cell internalization exhibit better endosomal escape and transfection efficiency.<sup>71–73</sup> Dense PEG coverage creates a steric hindrance for such conversion, inhibiting gene release. Several strategies to overcome the “dilemma” have been put into practice: enhancement of cellular uptake *via* ligand targeting (glycoproteins and antibodies) and

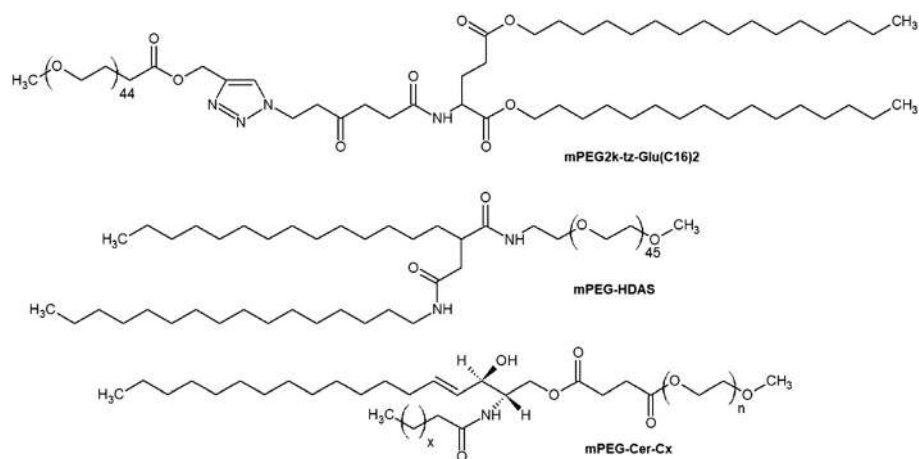


Fig. 6 Non-PE PEGylated lipids applied in the post-insertion method: mPEG2k-tz-Glu(C<sub>16</sub>)<sub>2</sub>, *N*-(methylpolyoxyethylene oxyethyl)-2-hexadecylcarbonylmethyl hexadecanoic amide (mPEG-HDAS), and mPEG-Cer-Cx. *N* – number of oxyethylene –CH<sub>2</sub>CH<sub>2</sub>O– units, *x* – number of homologous –CH<sub>2</sub>– groups.

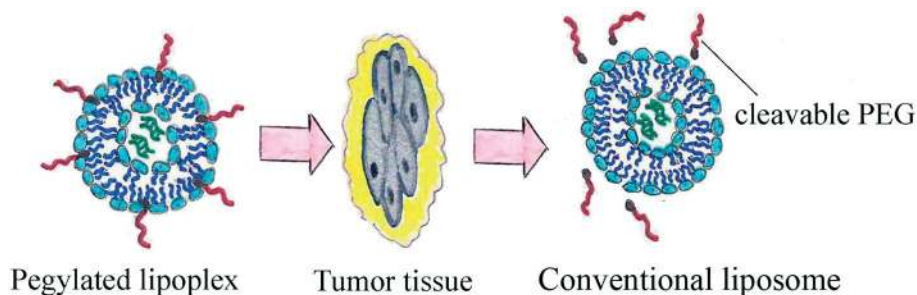


Fig. 7 PEG cleavage after tumor tissue penetration.

acceleration of endosomal escape *via* membrane fusion lipids, pH-sensitive cationic lipids (DODAP and DOTAP) that are activated by ionization and cleavable PEG-lipids.<sup>68,74</sup> The last one is supposed to turn into a “golden” standard of future PEGylated liposomes.<sup>75</sup>

### Cleavable PEGylated liposomes

The accelerated blood clearance (ABC) phenomenon describes a syndrome, in which the circulation time of the second dose administration of liposomes is significantly shorter than the first one, while their accumulation in the liver and spleen elevates. The immunogenicity of PEGylated liposomes creates a barrier for investigation of liposomal constructions and their clinical use. Antibody production significantly reduces drug effectiveness and safety. Currently, the ABC phenomenon is thought to be related to the anti-PEG IgM, which is produced in the spleen in response to the first injected dose.<sup>24,26,76</sup> Then the complement system is activated and the serum factor binds to PEG chains on the liposome periphery selectively. Thereby, the second dose of the administered drug is captured by Kupffer cells and a circulation time decrease is observed.

The use of cleavable PEG-lipids was developed as an innovative strategy for the usage of stealth liposomes, due to their properties of removing PEG head groups after cell penetra-

tion (Fig. 7). The PEG motif can be attached to the lipid anchor through ester, vinyl ester, hydrazone or disulfide bonds. Synthesis of those kinds of compounds (Fig. 8) is quite simple. It was shown that cleavable PEG derivatives dissociate from the particle periphery after penetration into tumor tissues and the extracellular area. The ABC phenomenon is eliminated or diminished as PEGs are not present in the complement system causing no immune response. The cleavage can be triggered by the temperature or pH level of the surrounding area; the labile bonds of cleavable PEG-lipids are very sensitive to the enzyme action and oxidation process.<sup>75</sup> PEG derivative cleavage does not cause the emergence of liposomal membrane damage; thus, cleavable PEG-lipids favorably differ from non-cleavable ones. It has been shown that the distribution in the liver and spleen and the blood clearance speed of cleavable PEG-liposomes after repeated administration remained at a constant level.<sup>77</sup> The cleavage process occurs very selectively in diseased tissue due to its unusual properties. For example, the extracellular area of solid tumors has a lower pH value than other body media or hyperthermia is induced in inflamed tissues caused by pyrogen action.

The higher the PEG cleavage degree, the less severe the occurrence of the ABC phenomenon. Therefore, mPEG-hz-CHEMS shows a lower ABC compared to mPEG-Chol and mPEG-DSPE. Disulfide bonds in mPEG-S-S-DSPE<sup>66</sup> and

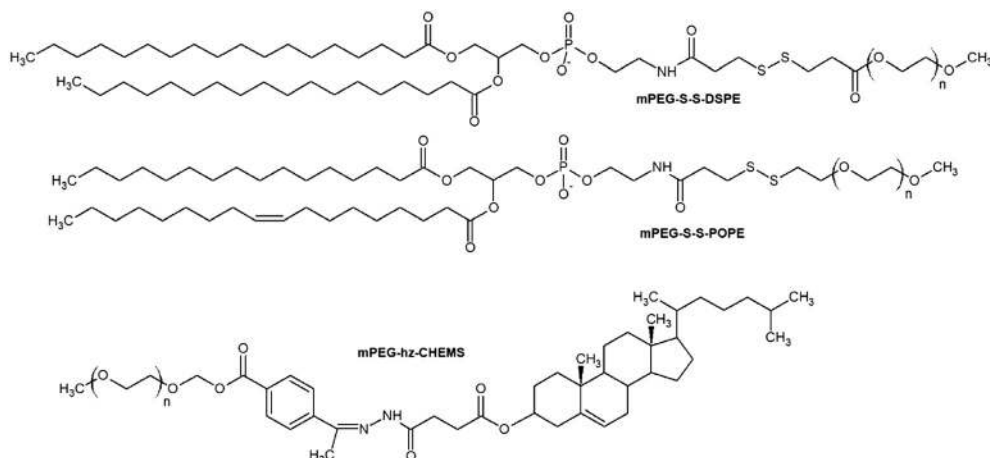


Fig. 8 Cleavable PEGylated lipids: mPEG-S-S-DSPE, mPEG-S-S-POPE, mPEG-hz-CHEMS. N – number of oxyethylene  $-\text{CH}_2\text{CH}_2\text{O}-$  units.

mPEG-S-S-POPE<sup>78</sup> lipids are destroyed in the presence of proteolytic enzymes (glutathione)/cysteine. Thus unshielded lipoplexes can escape from endosomes rapidly and release the drug fully until it would be disrupted in lysosomes.

## Conclusion

To reach a higher transfection effect, RNAs have to be packed into cationic liposomes. PEGylation increases the circulation time and tissue permeability of cationic liposomes. PEG-modified liposomes are able to deliver siRNA. There are two methods of PEG-based modification: pre- and post-insertion; the latter is preferable for considerable addition of PEG derivatives and helps to load bigger doses of the transferred material. PEGylation decreases the cellular uptake of liposomes and endosomal escape, the so-called “PEG dilemma”. To solve these problems, cleavable PEGylated liposomes were elaborated. Further investigations of the immunogenic properties of PEG coating, its density, the presence of additional functional groups, dosage frequency, *etc.* should be carried out.

## Conflicts of interest

There are no conflicts of interest to declare.

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## References

- 1 A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature*, 1998, **391**, 806–811.
- 2 M. R. Khaitov, I. P. Shilovskiy, A. A. Nikonova, N. N. Shershakova, O. Y. Kamyshnikov and A. A. Babakhin, *et al.*, Small Interfering RNAs Targeted to Interleukin-4 and Respiratory Syncytial Virus Reduce Airway Inflammation in a Mouse Model of Virus-Induced Asthma Exacerbation, *Hum. Gene Ther.*, 2014, **25**(7), 642–650, Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24655063>.
- 3 U. A. Ashfaq, M. Z. Yousaf, M. Aslam, R. Ejaz, S. Jahan and O. Ullah, siRNAs: Potential therapeutic agents against Hepatitis C Virus, *Virology*, 2011, **8**, 276–282, Available from: <http://virologyj.biomedcentral.com/articles/10.1186/1743-422X-8-276>.
- 4 D. Haussecker, Current issues of RNAi therapeutics delivery and development, *J. Controlled Release*, 2014, **195**, 49–54, Available from: <http://dx.doi.org/10.1016/j.jconrel.2014.07.056>.
- 5 J. A. H. Hoerter and N. G. Walter, Chemical modification resolves the asymmetry of siRNA strand degradation in human blood serum, *RNA*, 2007, **13**, 1887–1893, Available from: <http://www.rnajournal.org/cgi/doi/10.1261/rna.602307>.
- 6 S. Spagnou, A. D. Miller and M. Keller, Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA, *Biochemistry*, 2004, **43**, 13348–13356.
- 7 O. O. Koloskova, A. S. Nosova, Y. L. Sebyakin, A. A. Ilyukhina, I. P. Shilovskiy and M. R. Khaitov, Liposomal siRNA delivery systems (review), *Russ. J. Biopharm.*, 2017, **9**(5), 3–10.
- 8 E. A. Turetskiy, O. O. Koloskova, A. S. Nosova, I. P. Shilovskiy, Y. L. Sebyakin and M. R. Khaitov, Physicochemical properties of lipopeptide-based liposomes and their complexes with siRNA, *Biomed. Khim.*, 2017, **63**(5), 472–475.
- 9 A. T. Reibel, S. S. Müller, S. Pektor, M. Miederer, H. Frey and F. Roesch, Fate of Linear and Branched Polyether-Lipids in vivo in Comparison to their Liposomal Formulations by F-Radiolabeling and Positron Emission Tomography, *Biomacromolecules*, 2015, **16**(3), 842–851.
- 10 D. E. Owens and N. A. Peppas, Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, *Int. J. Pharm.*, 2006, **307**(1), 93–102.
- 11 S. Zalipsky and J. M. Harris, *Introduction to Chemistry and Biological Applications of Poly (ethylene glycol)*. In: *In Poly(ethylene glycol)*, 1997, pp. 1–13, Available from: [http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Introduction+to+Chemistry+and+Biological+Applications+of+Poly\(ethylene+glycol\)#1](http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Introduction+to+Chemistry+and+Biological+Applications+of+Poly(ethylene+glycol)#1).
- 12 F. M. Veronese and G. Pasut, PEGylation, successful approach to drug delivery, *Drug Discovery Today*, 2005, **10**(21), 1451–1458.
- 13 T. M. Allen and A. Chonn, Large unilamellar liposomes with low uptake into the reticuloendothelial system, *FEBS Lett.*, 1987, **223**(1), 42–46.
- 14 A. Gabizon, D. C. Price, J. Huberty and R. S. Bresalier, Effect of Liposome Composition and Other Factors on the Targeting of Liposomes to Experimental Tumors: Biodistribution and Imaging Studies, *Cancer Res.*, 1990, **50**(19), 6371–6378.
- 15 D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthey and S. K. Huang, *et al.*, Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**(24), 11460–11464, Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=53155&tool=pmcentrez&rendertype=abstract>.
- 16 A. L. Klivanov, K. Maruyama, V. P. Torchilin and L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, *FEBS Lett.*, 1990, **268**(1), 235–237.
- 17 G. Blume and G. Cevc, Liposomes for the sustained drug release in vivo, *Biochim. Biophys. Acta*, 1990, **1029**(1), 91–97, Available from: <http://linkinghub.elsevier.com/retrieve/pii/000527369090440Y>.
- 18 A. Mori, A. L. Klivanov, V. P. Torchilin and L. Huang, Influence of the steric barrier activity of amphipathic

- poly(ethyleneglycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo, *FEBS Lett.*, 1991, 284(2), 263–266.
- 19 T. M. Allen, C. Hansen, F. Martin, C. Redemann and A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, *Biochim. Biophys. Acta, Biomembr.*, 1991, 1066(1), 29–36.
  - 20 A. S. Nosova, O. O. Koloskova, Y. L. Sebyakin and M. R. Khaitov, Evolution of sterically stabilized liposome design modified with polyethyleneglycole (review), *Russ. J. Biopharm.*, 2018, 10, 3–9.
  - 21 J. A. Hubbell, S. P. Massia, N. P. Desai and P. D. Drumheller, Endothelial Cell-Selective Materials for Tissue Engineering in the Vascular Graft Via a New Receptor, *Nat. Biotechnol.*, 1991, 9(6), 568–572, Available from: <http://www.nature.com/doi/10.1038/nbt0691-568>.
  - 22 B. Hong, J. S. Brockenbrough, P. Wu and J. P. Aris, Nop2p is required for pre-rRNA processing and 60S ribosome subunit synthesis in yeast, *Mol. Cell. Biol.*, 1997, 17(1), 378–388.
  - 23 R. N. Majzoub, K. K. Ewert, E. L. Jacovetty, B. Carragher, C. S. Potter and Y. Li, *et al.*, Patterned Threadlike Micelles and DNA-Tethered Nanoparticles: A Structural Study of PEGylated Cationic Liposome-DNA Assemblies, *Langmuir*, 2015, 31(25), 7073–7083.
  - 24 T. Ishida, M. Ichihara, X. Y. Wang, K. Yamamoto, J. Kimura and E. Majima, *et al.*, Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes, *J. Controlled Release*, 2006, 112(1), 15–25.
  - 25 M. Hadjidemetriou, Z. Al-Ahmady and K. Kostarelos, Time-evolution of in vivo protein corona onto blood-circulating PEGylated liposomal doxorubicin (DOXIL) nanoparticles, *Nanoscale*, 2016, 8(13), 6948–6957.
  - 26 T. Ishida, M. Ichihara, X. Wang and H. Kiwada, Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes, *J. Controlled Release*, 2006, 115(3), 243–250.
  - 27 K. Shiraishi, K. Kawano, Y. Maitani, T. Aoshi, K. J. Ishii and Y. Sanada, *et al.*, Exploring the relationship between anti-PEG IgM behaviors and PEGylated nanoparticles and its significance for accelerated blood clearance, *J. Controlled Release*, 2016, 234, 59–67, Available from: <http://dx.doi.org/10.1016/j.jconrel.2016.05.010>.
  - 28 V. P. Torchilin and V. Weissing, *Liposomes: a practical approach*, 2003, p. 424.
  - 29 M. Kapoor and D. J. Burgess, Efficient and safe delivery of siRNA using anionic lipids: Formulation optimization studies, *Int. J. Pharm.*, 2012, 432(1–2), 80–90, Available from: <http://dx.doi.org/10.1016/j.ijpharm.2012.04.058>.
  - 30 A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini and N. Hossain, *et al.*, A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, *Nat. Biotechnol.*, 2008, 26(5), 561–569.
  - 31 N. F. Bouxsein, C. S. McAllister, K. K. Ewert, C. E. Samuel and C. R. Safinya, Structure and gene silencing activities of monovalent and pentavalent cationic lipid vectors complexed with siRNA, *Biochemistry*, 2007, 46(16), 4785–4792.
  - 32 J. Halder, A. A. Kamat, C. N. Landen, L. Y. Han, S. K. Lutgendorf and Y. G. Lin, *et al.*, Focal adhesion kinasetargeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy, *Clin. Cancer Res.*, 2006, 12(16), 4916–4924.
  - 33 K. A. Whitehead, J. R. Dorkin, A. J. Vegas, P. H. Chang, O. Veiseh and J. Matthews, *et al.*, Degradable lipid nanoparticles with predictable in vivo siRNA delivery activity, *Nat. Commun.*, 2014, 5, 1–10, Available from: <http://dx.doi.org/10.1038/ncomms5277>.
  - 34 V. Fehring, U. Schaeper, K. Ahrens, A. Santel, O. Keil and M. Eisermann, *et al.*, Delivery of therapeutic siRNA to the lung endothelium via novel Lipoplex formulation DACC, *Mol. Ther.*, 2014, 22(4), 811–820, Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3982492&tool=pmcentrez&rendertype=abstract>.
  - 35 P. E. Saw, J. Park, E. Lee, S. Ahn, J. Lee and H. Kim, *et al.*, Effect of PEG Pairing on the Efficiency of Cancer- Targeting Liposomes, *Theranostics*, 2015, 5(7), 9–14.
  - 36 H. Nakamura, A. S. Abu, M. Nishio, M. Tanaka, H. Ando and H. Kiwada, *et al.*, Intra-tumor distribution of PEGylated liposome upon repeated injection : No possession by prior dose, *J. Controlled Release*, 2015, 220, 406–413, Available from: <http://dx.doi.org/10.1016/j.jconrel.2015.11.002>.
  - 37 Y. Yang, Y. Yang, X. Xie, X. Cai, H. Zhang and W. Gong, *et al.*, Biomaterials PEGylated liposomes with NGR ligand and heat-activable cell- penetrating peptide e doxorubicin conjugate for tumor-speci fi c therapy, *Biomaterials*, 2014, 35(14), 4368–4381, Available from: <http://dx.doi.org/10.1016/j.biomaterials.2014.01.076>.
  - 38 K. Sou, T. Endo, S. Takeoka and E. Tsuchida, Poly (ethylene glycol) -Modification of the Phospholipid Vesicles by Using the Spontaneous Incorporation of Poly (ethylene glycol) -Lipid, *Bioconjugate Chem.*, 2000, 11, 372–379.
  - 39 R. N. Majzoub, C. L. Chan, K. K. Ewert, B. F. B. Silva, K. S. Liang and E. L. Jacovetty, *et al.*, Uptake and transfection efficiency of PEGylated cationic liposome-DNA complexes with and without RGD-tagging, *Biomaterials*, 2014, 35(18), 4996–5005.
  - 40 D. Pozzi, V. Colapicchioni, G. Caracciolo, S. Piovesana, A. L. Capriotti and S. Palchetti, *et al.*, Effect of polyethyleneglycol (PEG) chain length on the bio-nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells, *Nanoscale*, 2014, 6(5), 2782–2792, Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24463404>.
  - 41 J. Tang, R. Kuai, W. Yuan, L. Drake, J. J. Moon and A. Schwendeman, Effect of size and pegylation of liposomes and peptide-based synthetic lipoproteins on tumor targeting, *Nanomedicine*, 2017, 13(6), 1869–1878, Available from: <http://dx.doi.org/10.1016/j.nano.2017.04.009>.
  - 42 R. Dong, R. Liu, P. R. J. Gaffney, M. Schaeper-toens, P. Marchetti and C. M. Williams, *et al.*, Sequence-defined

- multifunctional polyethers via liquid-phase synthesis with molecular sieving, *Nat. Chem.*, 2018, **11**(2), 136–145, Available from: <https://doi.org/10.1038/s41557-018-0169-6>.
- 43 H. Xing, J. Li, W. Xu, K. Hwang, P. Wu and Q. Yin, *et al.*, The Effects of Spacer Length and Composition on Aptamer-Mediated Cell-Specific Targeting with Nanoscale PEGylated Liposomal Doxorubicin, *ChemBioChem*, 2016, **17**(12), 1111–1117, Available from: <http://doi.wiley.com/10.1002/cbic.201600092>.
- 44 P. A. Puchkov, I. A. Kartashova, E. V. Shmendel, A. S. Luneva, N. G. Morozova and M. A. Zenkova, *et al.*, Spacer structure and hydrophobicity influences transfection activity of novel polycationic gemini amphiphiles, *Bioorg. Med. Chem. Lett.*, 2017, **27**(15), 3284–3288, Available from: <http://dx.doi.org/10.1016/j.bmcl.2017.06.026>.
- 45 L. Y. Song, Q. F. Ahkong, Q. Rong, Z. Wang, S. Ansell and M. J. Hope, *et al.*, Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes, *Biochim. Biophys. Acta, Biomembr.*, 2002, **1558**(1), 1–13.
- 46 D. Zhi, S. Zhang, B. Wang, Y. Zhao, B. Yang and S. Yu, Transfection efficiency of cationic lipids with different hydrophobic domains in gene delivery, *Bioconjugate Chem.*, 2010, **21**(4), 563–577.
- 47 O. O. Koloskova, A. A. Nikonova, U. A. Budanova, I. P. Shilovskiy, I. A. Kofiadi and A. V. Ivanov, *et al.*, Synthesis and evaluation of novel lipopeptide as a vehicle for efficient gene delivery and gene silencing, *Eur. J. Pharm. Biopharm.*, 2016, **102**, 159–167, Available from: <http://dx.doi.org/10.1016/j.ejpb.2016.03.014>.
- 48 O. O. Koloskova, A. M. Gileva, M. G. Drozdova, M. V. Grechihina, N. E. Suzina and U. A. Budanova, *et al.*, Effect of lipopeptide structure on gene delivery system properties: Evaluation in 2D and 3D in vitro models, *Colloids Surf., B*, 2018, **167**, 328–336, Available from: <https://doi.org/10.1016/j.colsurfb.2018.04.003>.
- 49 Y. Hattori, Y. Machida, M. Honda, N. Takeuchi, Y. Yoshiike and H. Ohno, *et al.*, Small interfering RNA delivery into the liver by cationic cholesterol derivative-based liposomes, *J. Liposome Res.*, 2016, **27**(4), 264–273, Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27345333>.
- 50 M. L. Hensley, B. Hoppe, L. Leon, P. Sabbatini, C. Aghajanian and D. Chi, *et al.*, The costs and efficacy of liposomal doxorubicin in platinum-refractory ovarian cancer in heavily pretreated patients, *Gynecol. Oncol.*, 2001, **82**(3), 464–469, Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11520141>.
- 51 P. P. Wibroe, D. Ahmadvand, M. Ali, A. Yaghmur and S. M. Moghimi, An integrated assessment of morphology, size, and complement activation of the PEGylated liposomal doxorubicin products Doxil®, *J. Controlled Release*, 2016, **221**, 1–8, Available from: <http://dx.doi.org/10.1016/j.jconrel.2015.11.021>.
- 52 A. H. Sarris, F. Hagemester, J. Romaguera, M. A. Rodriguez, P. McLaughlin and A. M. Tsimberidou, *et al.*, Original article Liposomal vincristine in relapsed non-Hodgkin's lymphomas: Early results of an ongoing phase II trial, *Ann. Oncol.*, 2000, **11**, 69–72.
- 53 T. M. Allen and P. R. Cullis, Liposomal drug delivery systems: From concept to clinical applications, *Adv. Drug Delivery Rev.*, 2013, **65**(1), 36–48, Available from: <http://dx.doi.org/10.1016/j.addr.2012.09.037>.
- 54 P. S. Uster, T. M. Allen, B. E. Daniel, C. J. Mendez, M. S. Newman and G. Z. Zhu, Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time, *FEBS Lett.*, 1996, **386**(2–3), 243–246.
- 55 L. Nobs, F. Buchegger, R. Gurny and E. Allemann, Current methods for attaching targeting ligands to liposomes and nanoparticles, *J. Pharm. Sci.*, 2004, **93**(8), 1980–1992.
- 56 V. D. Awasthi, D. Garcia, R. Klipper, B. A. Goins and W. T. Phillips, Neutral and anionic liposome-encapsulated hemoglobin: effect of postinserted poly(ethylene glycol)-distearoylphosphatidylethanolamine on distribution and circulation kinetics, *J. Pharmacol. Exp. Ther.*, 2004, **309**(1), 241–248, Available from: <http://www.ncbi.nlm.nih.gov/pubmed/14718581%5Cnhttp://jpet.aspetjournals.org/content/309/1/241.full.pdf>.
- 57 C. C. Visser, S. Stevanović, L. H. Voorwinden, L. Van Bloois, P. J. Gaillard and M. Danhof, *et al.*, Targeting liposomes with protein drugs to the blood-brain barrier in vitro, *Eur. J. Pharm. Sci.*, 2005, **25**(2–3), 299–305.
- 58 T. Steenpaß, A. Lung and R. Schubert, TRESYLATED PEG-sterols for coupling of proteins to preformed plain or PEGylated liposomes, *Biochim. Biophys. Acta, Biomembr.*, 2006, **1758**(1), 20–28.
- 59 O. K. Nag, V. R. Yadav, A. Hedrick and V. Awasthi, Post-modification of preformed liposomes with novel non-phospholipid poly(ethylene glycol)-conjugated hexadecylcarbamoylmethyl hexadecanoic acid for enhanced circulation persistence in vivo, *Int. J. Pharm.*, 2013, **446**(1–2), 119–129, Available from: <http://dx.doi.org/10.1016/j.ijpharm.2013.02.026>.
- 60 A. C. N. Oliveira, K. Raemdonck, T. Martens, K. Rombouts, R. Simon-Vazquez and C. Botelho, *et al.*, Stealth monoolein-based nanocarriers for delivery of siRNA to cancer cells, *Acta Biomater.*, 2015, **25**, 216–229, Available from: <http://dx.doi.org/10.1016/j.actbio.2015.07.032>.
- 61 N. Golkar, A. M. Tamaddon and S. M. Samani, Effect of lipid composition on incorporation of trastuzumab-PEG-lipid into nanoliposomes by post-insertion method: physicochemical and cellular characterization, *J. Liposome Res.*, 2015, **26**(2), 113–125, Available from: <http://dx.doi.org/10.3109/08982104.2015.1048692>.
- 62 D. Belletti, G. Tosi, F. Forni, I. Lagreca, P. Barozzi and F. Pederzoli, *et al.*, PEGylated siRNA lipoplexes for silencing of BLIMP-1 in Primary Effusion Lymphoma: In vitro evidences of antitumoral activity, *Eur. J. Pharm. Biopharm.*, 2016, **99**, 7–17, Available from: <http://dx.doi.org/10.1016/j.ejpb.2015.11.007>.
- 63 F. S. Hassane, B. Frisch and F. Schuber, Targeted liposomes: Convenient coupling of ligands to preformed vesicles using “click chemistry”, *Bioconjugate Chem.*, 2006, **17**(3), 849–854.



- 64 A. Kumar, U. J. Erasquin, G. Qin, K. Li and C. Cai, "Clickable", polymerized liposomes as a versatile and stable platform for rapid optimization of their peripheral compositions, *Chem. Commun.*, 2010, 46(31), 5746–5748.
- 65 O. O. Koloskova, Y. G. Borodin, U. A. Budanova and Y. L. Sebyakin, Construction of doxorubicin sterically stabilized liposomal form performing the surface modification by means of 1,3-dipolar cycloaddition, *Russ. J. Biopharm.*, 2010, 2(6), 16–20.
- 66 L. Mei, L. Fu, K. Shi, Q. Zhang, Y. Liu and J. Tang, *et al.*, Increased tumor targeted delivery using a multistage liposome system functionalized with RGD, TAT and cleavable PEG, *Int. J. Pharm.*, 2014, 468(1–2), 26–38, Available from: <http://dx.doi.org/10.1016/j.ijpharm.2014.04.008>.
- 67 K. K. Ewert, V. R. Kotamraju, R. N. Majzoub, V. M. Steffes, E. A. Wonder and T. Teesalu, *et al.*, Synthesis of linear and cyclic peptide-PEG-lipids for stabilization and targeting of cationic liposome-DNA complexes, *Bioorg. Med. Chem. Lett.*, 2016, 26(6), 1618–1623, Available from: <http://dx.doi.org/10.1016/j.bmcl.2016.01.079>.
- 68 Y. Xia, J. Tian and X. Chen, Effect of surface properties on liposomal siRNA delivery, *Biomaterials*, 2016, 79, 56–68, Available from: <http://dx.doi.org/10.1016/j.biomaterials.2015.11.056>.
- 69 A. Lechanteur, T. Furst, B. Evrard, P. Delvenne, P. Hubert and G. Piel, PEGylation of lipoplexes: The right balance between cytotoxicity and siRNA effectiveness, *Eur. J. Pharm. Sci.*, 2016, 93, 493–503, Available from: <http://dx.doi.org/10.1016/j.ejps.2016.08.058>.
- 70 J. Smisterova, A. Wagenaar, M. C. A. Stuart, E. Polushkin, G. Brinke and R. Hulst, *et al.*, Molecular Shape of the Cationic Lipid Controls the Structure of Cationic Lipid/Dioleoylphosphatidylethanolamine-DNA Complexes and the Efficiency of Gene Delivery, *J. Biol. Chem.*, 2001, 276(50), 47615–47622.
- 71 N. Dan and D. Danino, Structure and kinetics of lipid – nucleic acid complexes, *Adv. Colloid Interface Sci.*, 2014, 205, 230–239, Available from: <http://dx.doi.org/10.1016/j.cis.2014.01.013>.
- 72 I. Koltover, T. Salditt and J. O. Ra, An Inverted Hexagonal Phase of Cationic Liposome – DNA Complexes Related to DNA Release and Delivery, *Science*, 1998, 281, 78–81.
- 73 J. O. Rädler, J. O. Ra, I. Koltover, T. Salditt and C. R. Safinya, Structure of DNA – Cationic Liposome Complexes : DNA Intercalation in Multilamellar Membranes in Distinct Interhelical Packing Regimes, *Science*, 1997, 275, 810–814.
- 74 H. Hatakeyama, H. Akita and H. Harashima, The Polyethyleneglycol Dilemma: Advantage and Disadvantage of PEGylation of Liposomes for Systemic Genes and Nucleic Acids Delivery to Tumors, *Biol. Pharm. Bull.*, 2013, 36(6), 892–899, Available from: <http://jlc.jst.go.jp/DN/JST.JSTAGE/bpb/b13-00059?lang=en&from=CrossRef&type=abstract>.
- 75 Y. Fang, J. Xue, S. Gao, A. Lu, D. Yang and H. Jiang, *et al.*, Cleavable PEGylation: a strategy for overcoming the "PEG dilemma" in efficient drug delivery, *Drug Delivery*, 2017, 24(sup 1), 22–32, Available from: <https://doi.org/10.1080/10717544.2017.1388451>.
- 76 T. Ishida, M. Harada, Y. W. Xin, M. Ichihara, K. Irimura and H. Kiyada, Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: Effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes, *J. Controlled Release*, 2005, 105(3), 305–317.
- 77 H. Xu, K. Q. Wang, Y. H. Deng and D. W. Chen, Effects of cleavable PEG-cholesterol derivatives on the accelerated blood clearance of PEGylated liposomes, *Biomaterials*, 2010, 31(17), 4757–4763, Available from: <http://dx.doi.org/10.1016/j.biomaterials.2010.02.049>.
- 78 P. S. Kulkarni, M. K. Haldar, R. R. Nahire, P. Katti, A. H. Ambre and W. W. Muhonen, *et al.* MMP - 9 Responsive PEG Cleavable Nanovesicles for Efficient Delivery of Chemotherapeutics to Pancreatic Cancer, *Mol. Pharmaceutics*, 2014, 11, 2390–2399.