摘要

基因药物输递载体必须将携载的基因输递进入细胞质或细胞核中，才能发挥DNA 或 RNA 的作用。因此，合理设计核酸输递载体至关重要。与病毒载体相比，非病毒载体在基因治疗能够避免多种致命性感染。然而，非病毒载体转染效率低，严重制约了其发展。受病毒结构的启发以及对病毒感染过程认识的加深，许多具有仿生结构的非病毒载体被设计应用于提高基因的体内外转染效率。然而，效果甚微。本综述中，我们将研究基因治疗特别在肿瘤治疗中，仿病毒载体的研究发展；通过探讨载体材料结构及功能之间的关系，为构建应用于基因治疗领域的安全、高效非病毒载体提供指导。

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Research review paper

Design, preparation and application of nucleic acid delivery carriers

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ABSTRACT

Gene delivery vectors must deliver their cargoes into the cytosol or the nucleus, where DNA or siRNA functions *in vivo*. Therefore it is crucial for the rational design of the nucleic acid delivery carriers. Compared with viral vectors, non-viral vectors have overcome some fatal defections in gene therapy. Whereas the most important issue for the non-viral vectors is the low transfection efficiency, which hinders the progress of non-viral carriers. Sparked by the structures of the virus and understanding of the process of virus infection, various biomimic structures of non-viral carriers were designed and prepared to improve the transfection issues *in vitro* and *in vivo*. However, less impressive results are achieved. In this review, we will investigate the evolution of the virus-mimicking carriers of nucleic acids for gene therapy, especially in cancer therapy; explore and discuss the relationship between the structures, materials and functions of the carriers, to provide guidance for establishing safe and highly efficient non-viral carriers for gene therapy.

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1. Introduction

Nucleic acids, which include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), have many therapeutic applications. They could be used as pharmaceutical agents to treat diseases, involving correction of genetic defects and gene augmentation for chronic diseases including cancers. Herein, gene therapy with nucleic acids has attracted more and more attentions. However, it is generally difficult for the naked nucleic acid to deliver into cells of the body mainly due to enzymatic degradation by the nucleases (Takakura et al., 2001), which limit serum half-life of DNA to 10 min (Kawabata et al., 1995) and unmodified small interfering RNA (siRNA) to 5–60 min (Soutschek et al., 2004). Moreover, nucleic acids are negatively charged biomacromolecules, which hinder them across the cellular membranes without the aid of external force.

How to introduce foreign nucleic acid into cells? Researchers get the answers from the process of virus infection. Generally, viruses could enter the cells via endocytosis and release viral genome, which could replicate and transcribe in host cells. Then viral mRNAs are translated and proteins are processed to make multiple copies of viruses. Finally, viruses assemble inside the host cells and escape to the exterior (Fig. 1). Through understanding the virus infection process, researchers exploit the delivery carriers for nucleic acids. The viral carriers, like adenoviral vectors (Hartman et al., 2008) and retroviral/lentiviral vectors (Cooray et al., 2012; Ellis, 2005) were first used as they could bind to

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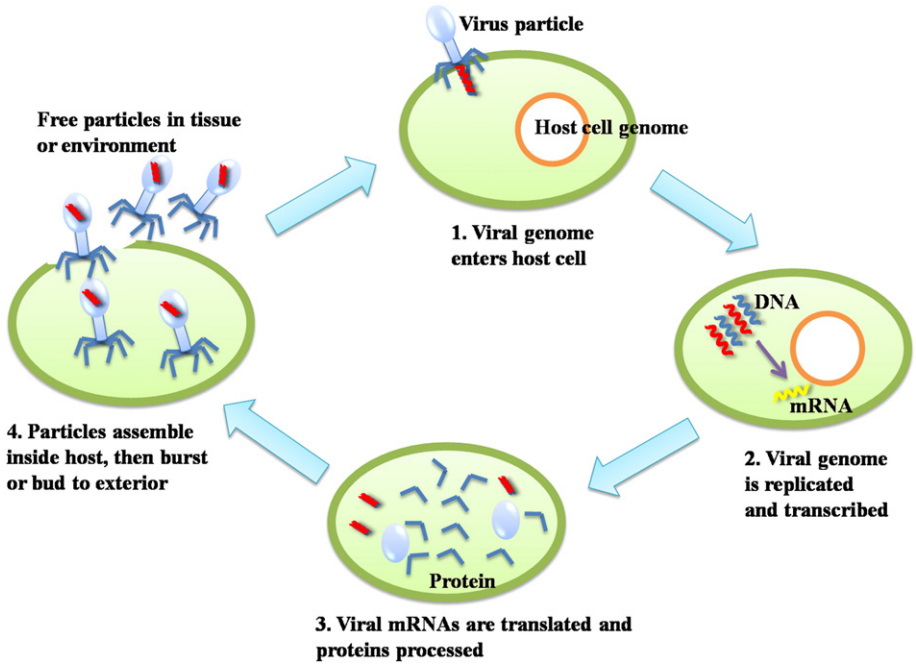

Fig. 1. The process of virus infection.

their hosts and introduce their genetic materials into the host cells. The viral vectors were prepared by removing the virus' own cargo and subsequently packaging the genes of interest into the viral shells (Selkirk, 2004). These viral delivery systems showed relatively high transfection efficiency both *in vitro* and *in vivo*. However, the death of an 18-year old participant in virus treatment using adenovirus vector to deliver therapeutic DNA to the liver in the University of Pennsylvania study resulted in a significant setback to gene therapy (Lehrman, 1999; Marshall, 1999). It revealed the risk of overwhelming inflammation from the virus treatment. Apart from the clinical safety issue (Check, 2005), the viral delivery systems exhibited some other critical problems, such as small cargo capacity, lack of long-term transgene expression, resistance to repeated administration, difficulty in large-scale pharmaceutical grade production and quality control and so on (Chowdhury, 2009; Collins et al., 2008), which led to a reconsideration for the use of viral vectors and accelerated the research on non-viral carriers since they could overcome toxicity issues for viral delivery.

In order to deliver therapeutic genes into the target cells safely and efficiently, it is especially crucial for the rational design of the non-viral delivery carriers. As we known, virus consists three parts, including the genetic materials like DNA or RNA, a protein coat that covers and protects these genes, and an envelope of lipids surrounding the protein coat. Sparked by the structures of the virus, many virus-mimicking non-viral carriers are developed for therapeutic applications. They exhibit low immunogenicity, low toxicity, ease of production, and the potential of transferring large pieces of nucleic acids into cells (Chen and Huang, 2008; Gary et al., 2007; Guo and Huang, 2012).

Besides the safety issues, the viral vectors have low specific targeting, like tumor-targeting in cancer therapy. Non-viral vectors have presented some crucial advantages over viral vectors to improve the toxicity and targeting problems by using nanotechnology in tumor tissues, naming the enhanced permeability and retention (EPR) effect with the characteristics of tumor vasculature (Fig. 2) (Iyer et al., 2006; Maeda et al., 2003; Torchilin, 2011). Non-viral vectors within nanoscale could pass through tumor blood vessels and get more accumulation in tumor tissues than in normal tissues. More importantly, to overcome the extracellular and intracellular barriers during the delivery of nucleic acids, various non-viral carriers have been designed and prepared. These carriers include lipids (Ewert et al., 2010; Gomes-da-Silva et al., 2012; Yang et al., 2011), biomaterials (e.g. chitosan, cyclodextran) (Li and Loh, 2008; Saranya et al., 2011), synthesized polymers (Martello et al., 2012; Patnaik and Gupta, 2013; Son et al., 2012) and dendrimers (Percec et al., 2010). But until now, only few of these vectors step into clinical trials or commercial availability. The most important issue for the non-viral vectors is the low transfection efficiency which hinders the progress of non-viral carriers.

For systemic administration of these non-viral vectors into target cells, there are many extracellular and intracellular barriers that must be overcome to get efficient transfection, including effective circulation in the blood, extravasation across the vascular endothelial membranes, diffusion through the extracellular matrix, cellular association and uptake, endosomal escape, unpacking of the complexes of nucleic acids and release of the intact therapeutic nucleic acids in the cytoplasm

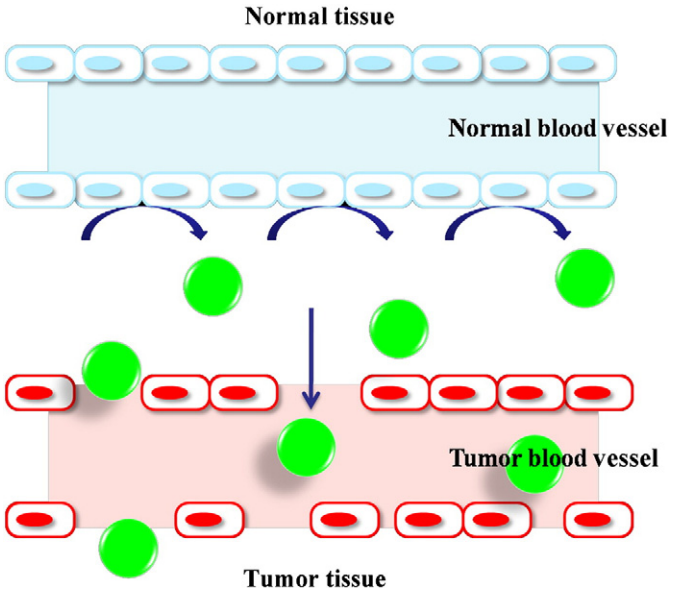

Fig. 2. Schematic representation of the passive tumor targeting of non-viral vectors and enhanced permeability and retention (EPR) effect.

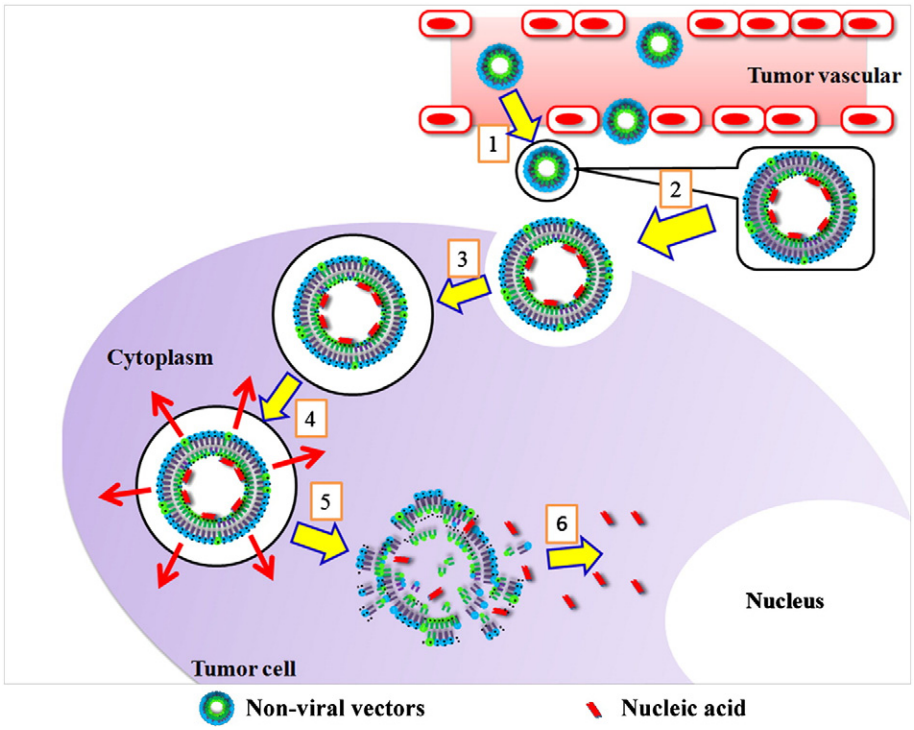

Fig. 3. Illustration of various barriers encountered by the nucleic acid-containing vectors upon intravenous injection. (1) Extravasation across the vascular endothelial membranes, (2) cellular association, (3) endocytosis, (4) and (5) endosomal escape, (6) release of nucleic acid.

(siRNA) or in the nucleus (DNA) (Fig. 3). Considering these, it is very significant for the rational design of the non-viral carriers. Virus could infect cells effectively, and the viral vectors exhibit superior performance on transfection. Whereas the non-viral vectors, which mimic the structures of virus, demonstrated less impressive performance in gene therapy, though they could overcome some fatal drawbacks of the viral vectors. Herein, in this review, we will focus on the evolution of the non-viral delivery carriers of nucleic acids for gene therapy, especially for cancer therapy; explore the relationship of structures, materials and functions of the non-viral gene delivery vectors; and discuss the influence of virus-mimicking nanocapsule structures on the functionality of non-viral vectors, to provide guidance for establishing safe and highly efficient non-viral carriers for gene therapy.

2. Liposome

Motivated by the structures of virus, lipids are used in the delivery vectors to cover and protect the cargo. Liposome was first used for drug delivery for cancer therapy in the 1960s, and it was not until 1995 that the delivery of anticancer drug had made remarkable progress. The first liposomal drug, Doxil (doxorubicin HCl liposome injection), was approved by the FDA to treat ovarian cancer, AIDS-related

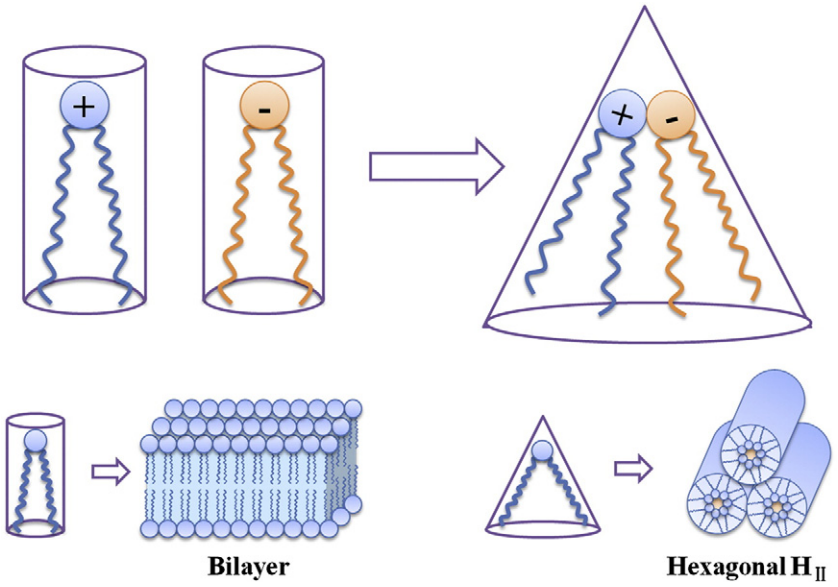

Fig. 4. Illustration of the proposed mechanism of membrane disruptive effects of cationic lipids. Cationic lipids and anionic lipids of endosomal membranes adopt a cylindrical molecular shape (lamellar phase, L_{α}), which supports bilayer structures. Whereas when cationic and anionic lipids are mixed together, they form a 'cone' shape, which is readily for the hexagonal phase (H_{II}) formation.

Kaposi's sarcoma, and multiple myeloma (Davis et al., 2008). And most recently, the FDA approved the first generic version of the drug Doxil to help alleviate shortages. For gene delivery, cationic lipids are introduced into delivery vectors based on the negative charge nature of the nucleic acids. Since Felgner et al. introduced cationic lipid (N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride, DOTMA) for DNA delivery *in vitro*, and facilitated the fusion of the lipoplexes with plasma membranes of tissue cells (Felgner et al., 1987), liposome-based non-viral carriers are currently used in gene therapy for *in vitro* and *in vivo* applications. However, their transfection efficiencies and silencing efficiencies remain low compared to those of viral vectors. This demonstrated the currently poor understanding of transfection-related mechanisms at the molecular and self-assembled levels, including a lack of knowledge about interactions between membranes and nucleic acids and between complexes and cellular components.

In most liposome-based vectors for nucleic acid delivery, there always have a cationic lipid and a neutral helper lipid. Cationic lipid is used to bind nucleic acids to protect them from nucleinase and facilitate the system's endocytosis. Moreover, they can form ion pairs with anionic phospholipids membranes to destabilize the membranes by changing formation of the lamellar phase (L_α) to inverted hexagonal phase (H_{II}) (Fig. 4) (Koltover et al., 1998; Liu and Huang, 2010), and improve the endosomal escape and release. The neutral lipid helps the systems endosomal escape by the aid of H_{II} formation. Herein, the internal structures of the lipids and the surface charge density of lipoplexes are crucial to design efficient liposome based gene vectors (Ewert et al., 2005; Ewert et al., 2010).

Safinya et al. synthesized multivalent cationic lipids with highly charged dendritic headgroups (MVLG1, MVLG2, MVLBisG1 and MVLBisG2, Fig. 5) to probe the structure–transfection efficiency relationships of cationic liposome–DNA complexes. The headgroup of the multivalent lipids had an ornithine-based core that is terminated by ornithine or carboxyspermine moieties, resulting in a maximum of +16 headgroup charge, which extended the range of membrane charge density for the liposomes. Complexes of DNA with the dendritic lipids and neutral 1,2-dioleoyl-*sn*-glycero phosphatidylcholine (DOPC) exhibited efficient transfection on the relevance of membrane charge density, and no significant cytotoxicity was observed for any of the complexes (Ewert et al., 2006a). When the composition of MVLBG2 was only 10 mol% in the delivery system, the transfection efficiency of the complexes surpassed that of the commercially available and optimized DOTAP (1,2-dioleoyl-3-trimethyl ammonium-propane)-based complexes in mouse L-cells, HeLa cells, 293 cells and mouse embryonic fibroblast cell lines (Fig. 6). Mouse embryonic fibroblast cell lines are

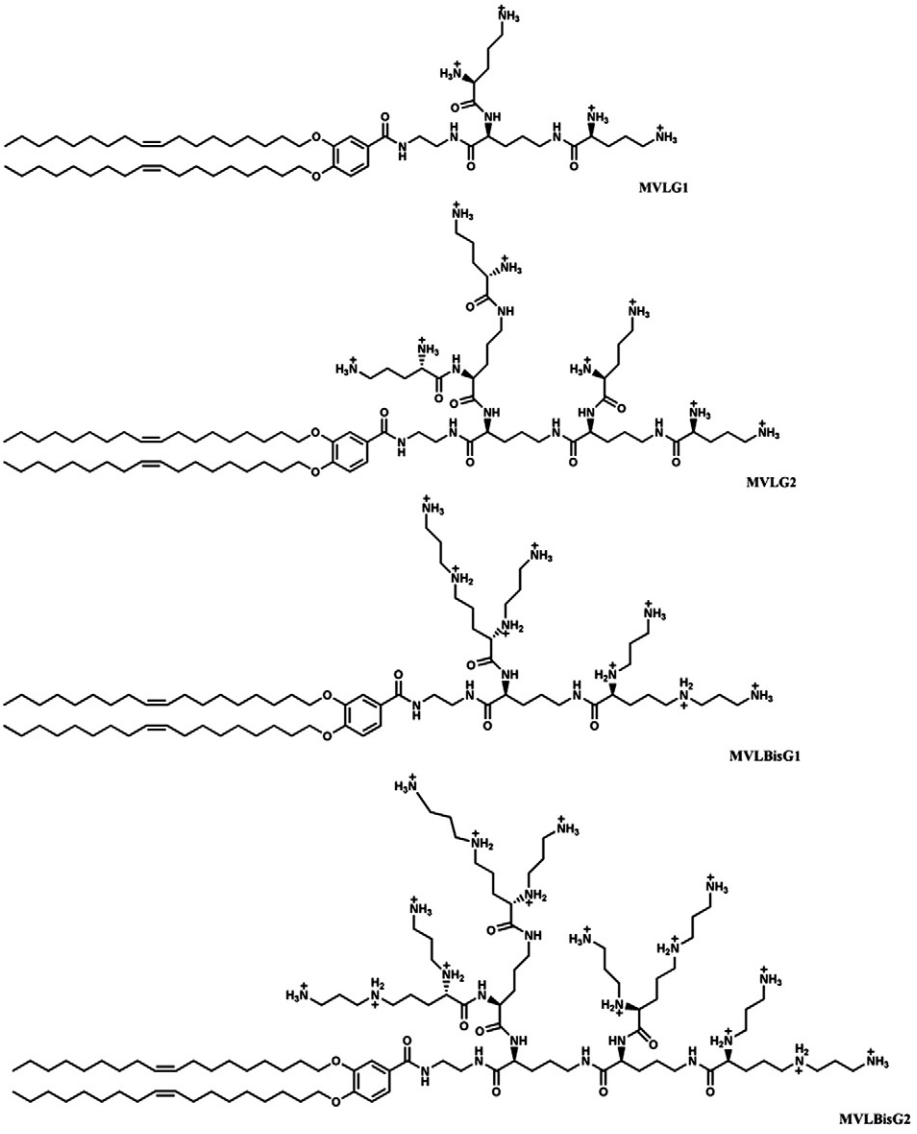

Fig. 5. Chemical structures and maximum charge of the MVLG1, MVLG2, MVLBisG1, and MVLBisG2.

known as "hard to transfect" cell lines (Ewert et al., 2006b). Moreover, the relatively small amount of highly charged cationic lipid for the delivery vectors resulted in a major reduction of the toxicity.

Miller et al. investigated a versatile cationic lipid, DODAG (Fig. 7), with guanidinium functional groups formulated with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), which appeared to mediate high transfection in OVCAR-3, IGROV-1 and HeLa cell lines in the presence or absence of serum. Moreover, DODAG alone formed lipoplex exhibited efficient delivery of anti-hepatitis B virus (HBV) siRNAs to the liver of HBV transgenic mice *in vivo* with few liver toxicity, resulting in a partial treatment of HBV infection over a period of 28 days, which was equivalent to the efficacy of a licensed antiviral drug, lamivudine (Fig. 8) (Mével et al., 2010). Semple and Akinc et al. designed and prepared a series of DLinDMA (1,2-dilinoleyloxy-3-dimethylaminopropane)-based lipids (Fig. 7) with superior delivery capacity by altering the headgroup and linker of the cationic lipid, and investigated the structures' effect on the efficacy and tolerability in a systematic fashion. The DLinDMA cationic lipids have a tertiary amine in the headgroup, which could keep neutral in physiological environment and change to cationic in the acidic condition of the endosome. This ionizable pH-responsive strategy could maintain the delivery vectors' stability in the circulation and endosomal escape after internalization. The best formulation with DLin-KC2-DMA exhibited *in vivo* activity at siRNA doses as low as 0.01 mg/kg in rodents and 0.1 mg/kg in nonhuman primates (Semple et al., 2010). Furthermore, it was investigated that the superior capacity of DLin-KC2-DMA in gene silencing was mainly due to higher cellular uptake and enhanced stimulation on siRNA release, compared with DLinDMA (Lin et al., 2013). The charge density and the spacer linker of the cationic lipids play a significant role in the extracellular and intracellular delivery of nucleic acids.

However, although the cationic lipids have brought us to a step closer to the goal of realizing nucleic acids' application to human disease, there are still some problems that we need to worry. For example, though the fusogenic nature of lipids in the liposomes helps to promote transfection *in vitro*, the lipoplexes are prone to be unstable to aggregate in complex *in vivo* environments, which results in the drug leakage during systemic administration. Considering their inherent instability, lipoplexes are generally prepared immediately before use, and set up many drawbacks on manufacture and drug administration.

3. Polymersome

Polymer carriers are promising vectors for gene delivery due to their potential of structural diversity and flexible functionality. They could enhance the complexes of nucleic acids' stability during the circulation and give a great imaging potential for researchers to reconstruct multifunctional vectors to overcome some defects of liposomes (Martello et al., 2012; Zhang et al., 2008). Over the years, a large number of cationic polymers as non-viral gene carriers have sparked the researchers' interests, including polypeptides (like poly(L-lysine)) (Choi et al., 1998; Hofman et al., 2013; Huang et al., 2010; Ohsaki et al., 2002; Zhang et al., 2010), polyethylenimine (PEI) (Akinc et al., 2005; Boussif et al., 1995; Namgung et al., 2009; Patnaik and Gupta, 2013), poly(β-amino esters) (PBAEs) (Shen et al., 2009; Zhang et al., 2013; Zugates et al., 2007) and so on (Huang et al., 2012; Li and Loh, 2008; Saranya et al., 2011; Zhang et al., 2008). They generally contain a high density of amine groups, which could be protonatable at physiological pH. When mixed with negatively charged nucleic acids, they can form stable complexes (polyplex) through electrostatic interaction and entropy change. However, the transfection efficiency and safety vary due to the structures of polymers (Peterca et al., 2011). Understanding how the structures of these polymer carriers affect the intercellular uptake and transfection will assist us in optimal design of more effective vectors for gene therapy.

3.1. Poly(L-lysine) (PLL)

The primary amino groups of lysine in poly(L-lysine) could be protonated in a physiological environment to electrostatically interact with negatively charged phosphate groups of DNA to form complexes. Though polylysines are biodegradable, they have high cationic toxicity which limits their applications in gene delivery. To reduce their toxicity, modifications were made for better applications in nucleic acid delivery. Comb-type poly(ethylene glycol)-graft-poly(L-lysine) (PEG-g-PLL) was first synthesized by Kim et al. with different PEG ratios. PEG-g-PLL with a 10 mol% PEG ratio demonstrated good efficient protection of DNA from enzymatic degradation and transfection efficiency in HepG2 cells, and maintenance of gene expression for up to 96 h (Choi et al., 1998). However, the PLL/DNA complexes showed a relatively high cytotoxicity

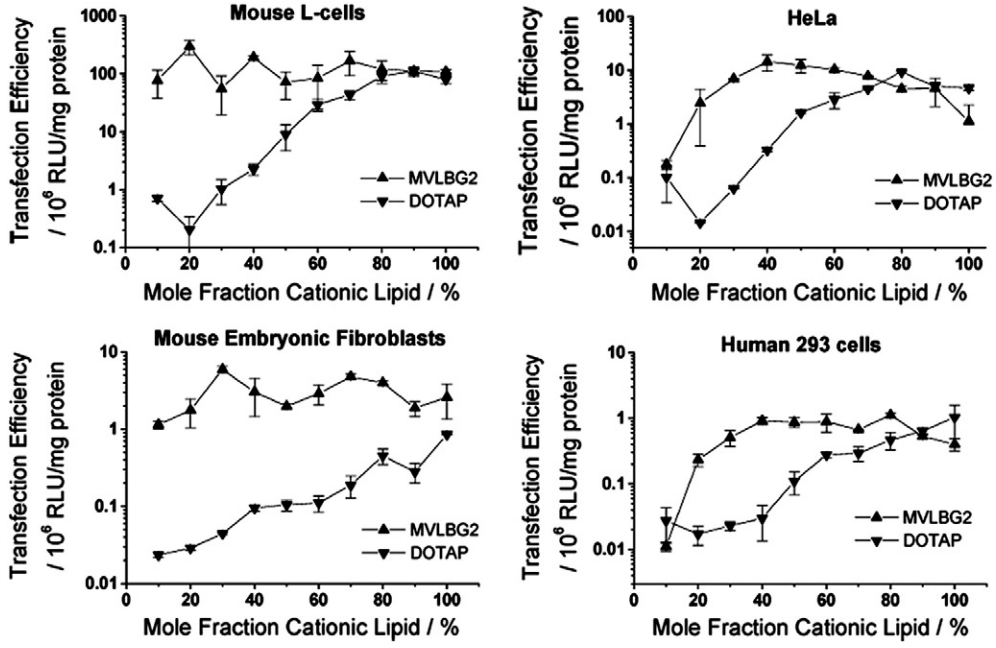

Fig. 6. Transfection efficiencies for DOTAP/DOPC and MVLBG2/DOPC complexes in four different cell lines. The data points were obtained at a constant lipid to DNA charge ratio ($\rho_{chg} = 7$ for HeLa cells and $\rho_{chg} = 4.5$ for others). Reproduced with permission from Ewert et al. (2006b). Copyright 2006 American Chemical Society.

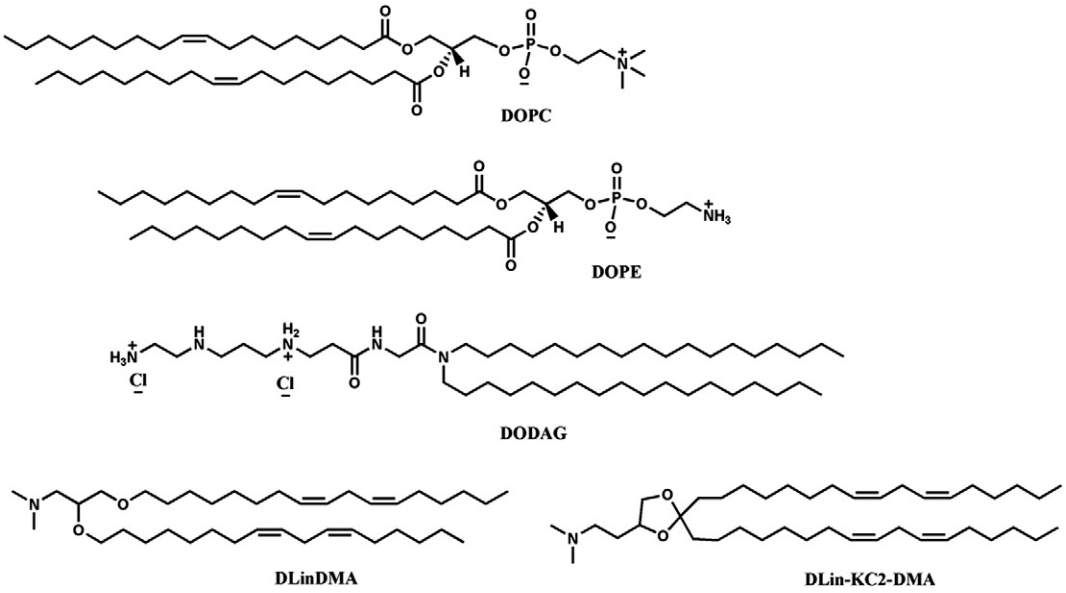

Fig. 7. Chemical structures of DOPC, DOPE, DODAG, DLinDMA and DLin-KC2-DMA.

and were prone to aggregate depending on the ionic strength (Liu et al., 2001).

Urtti et al. investigated the influence of various shapes and molecular weights, including linear, grafted, dendritic and branched, on the transfection efficacy of DNA delivery system based on poly(L-lysine) (Fig. 9). Most of the linear polymers were more efficient than the dendritic ones when condensed with DNA. PEGylation could increase the DNA condensation and transfection efficacy, but the overall transfection level of polylysines was low, which might be due to the inadequate escape of the complexes from endosomes or poor release of DNA from the complexes (Männistö et al., 2002). This may have a connection with the pKa of the PLL which influences the buffering capacity to disrupt endosomal membranes.

Comparing linear poly(L-lysine) (PLL) with dendritic poly(L-lysine) (KG6), Niidome et al. found that KG6-mediated gene expression was 100-fold higher than that mediated by PLL, though its DNA compact capacity and cellular uptake were only one-fourth as well as that with PLL (Fig. 10) (Yamagata et al., 2007). It seemed that the weakly compacted capacity of KG6 helped DNA escaping to the cytosol, whereas the PLL exhibited strong compaction with DNA, resulting in difficult dissociation from the vectors for gene expression. Later, four dendritic poly(L-lysines) generations were designed to investigate the generation effect on the transfection efficacy against Hep2 cells (Hofman et al., 2013), and higher generations (G3, G4, G5) were found to exhibit more effective transfection capacity in comparison with low generation G2 (Huang et al., 2010). Dendritic PLL G5 delivered the siRNA with the same efficiency as commercial Lipofectamine 2000, displaying excellent ability to bind DNA and internalize it into the cells.

3.2. Polyethylenimine (PEI)

PEI was first described for gene delivery purposes by Behr's lab (Boussif et al., 1995). It had primary, secondary and tertiary amines, of which two-thirds of the amines could be protonated in a physiological environment. The unprotonated amines with different pKa values conferred a buffering effect over a wide range of pH, which helped PEI escape from the endosome and facilitated the intracellular trafficking of nucleic acids (Akinc et al., 2005). Compared with PLL, PEI is more effective due to a high density of near-neutral pKa groups that can buffer the acidic environment of the endosome and facilitate endosomal escape with the help of the "proton sponge" effect (Patnaik and Gupta, 2013).

The capacity of PEI in gene delivery and cytotoxicity is critically influenced by its molecular weight. Thus, balance between transfection efficiency and toxicity should be considered for gene therapy. PEI with a high molecular weight (such as 25 kDa) displayed high transfection efficiency due to efficient endosomal escape, but relatively considerable

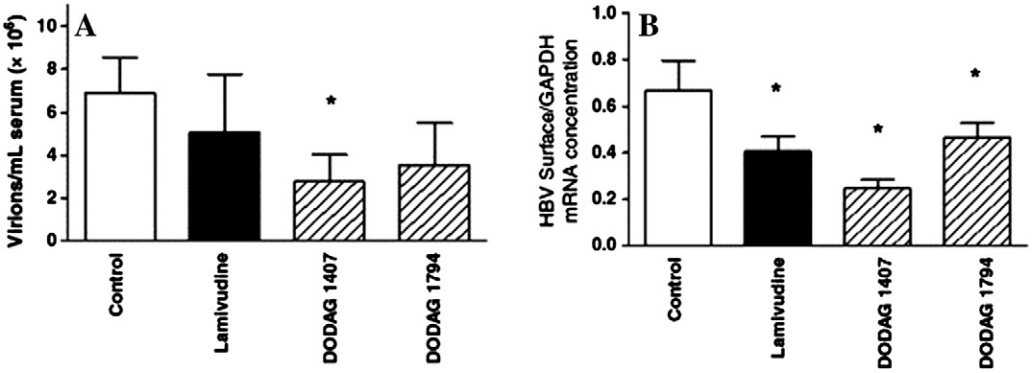

Fig. 8. (A) Circulating viral particle equivalent levels (VPEs) measured at 28 days in HBV transgenic mice treated every 3 days with a siRNA-DODAG formulation delivering non-functional control siRNA (Control), or siRNA-DODAG formulation delivery functional siRNA-1407 or siRNA-1794 (DODAG 1407 or DODAG1794 respectively), in comparison with lamivudine. (B) HBVs-antigen (HBsAg) mRNA levels measured at 28 days in HBV transgenic mice treated exactly as in (A). Reproduced with permission from Mével et al. (2010).

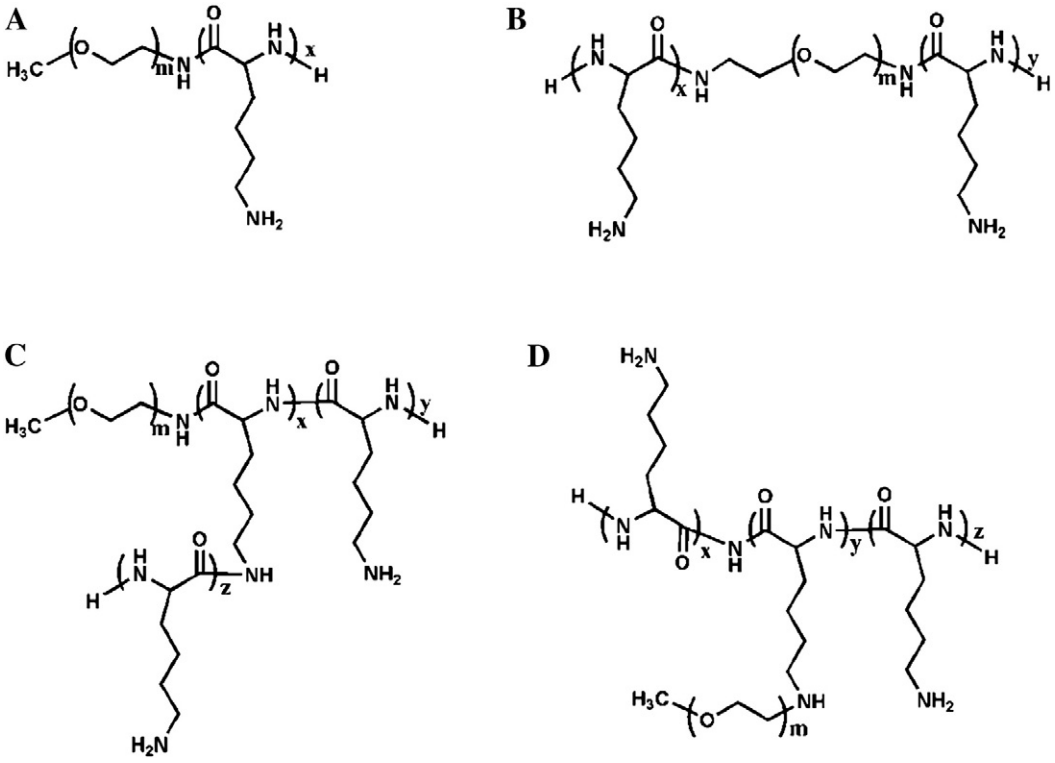

Fig. 9. Scheme of various PEGylation PLLs.

toxicity, whereas PEI with low molecular weight has low toxicity and less transfection efficiency (Fischer et al., 1999). In comparison with branched PEI, linear PEI seems to have shown excellent efficiency and low toxicity (Wightman et al., 2001). Mok and Nam et al. incorporated thiol-terminated siRNA with thiol-grafted linear PEI via disulfide bonds to achieve highly efficient gene silencing (Hong et al., 2013). The redox-

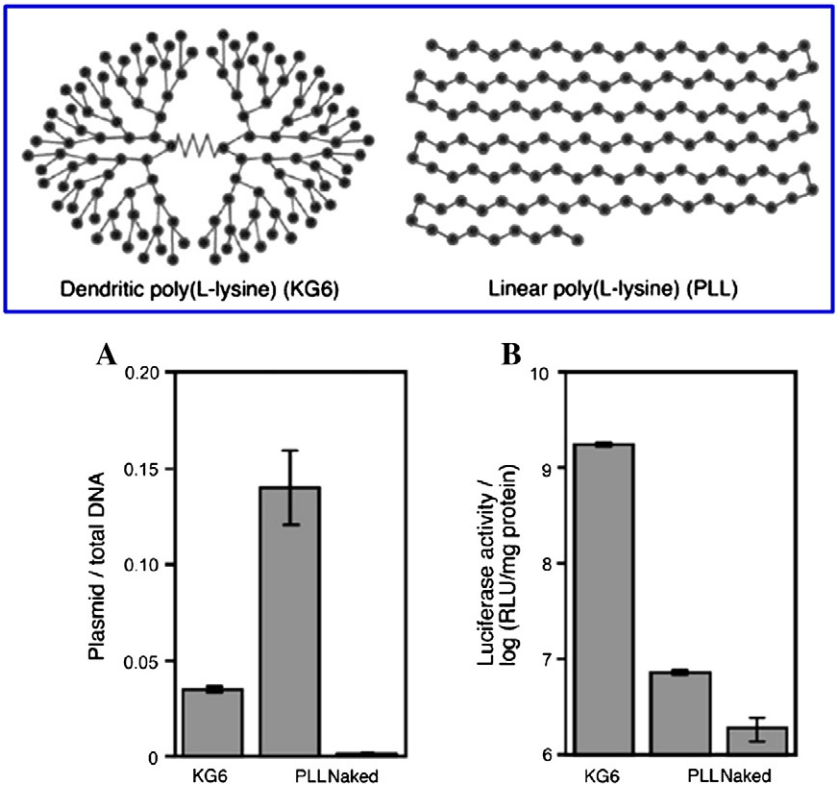

Fig. 10. Structures of dendritic poly(L-lysine) of the 6th generation (KG6) and linear poly(L-lysine) (PLL) (upper). (lower) DNA binding and uptake into cells, and transfection efficiencies mediated by KG6 and PLL. (A) Amounts of DNA binding and uptake into cells at an N/P ratio of 8 for 3 h at 37 °C. (B) Transfection efficiencies of KG6 or PLL into CHO cells. Cells were incubated with KG6 or PLL–DNA complexes at an N/P ratio of 8 for 3 h at 37 °C. Reproduced with permission from Yamagata et al. (2007).

crosslinked siRNA/LPEI complexes demonstrated excellent structural stability intracellular translocation whereas feasible to dissociate in a reductive condition.

To decrease the cytotoxicity of PEI, various modifications have been operated to optimize the PEI-based delivery vectors. In 2005, Feijen et al. introduced PEG into low molecular weight linear PEI to form triblock copolymers, and demonstrated that the transfection activity of polyplexes of PEI–PEG–PEI (4000–300–4000) showed transfection efficiency comparable to the polyplexes of branched PEI 25 kDa. Moreover, the PEI–PEG–PEI displayed an improved safety ability in comparison with high molecular weight PEIs (Zhong et al., 2005). Later, low molecular weight linear PEI (2.5 kDa) was conjugated with multiarm PEG (10 kDa) to form star-shaped copolymers, which imparted a relatively high charge density for the polyplexes. The special structures helped achieve higher transfection efficiency and prevented the unwanted aggregation of the smaller polyplex particles due to the low molecular weight of LPEI and multiarm PEG (Namgung et al., 2009). PEI-7 K-L, linked by short PEI chains (2 kDa) with dithiobis(succinimidyl propionate) (DSP), exhibited less cytotoxity and is 2–10 times more effective than PEI-25 K and Lipofectamine 2000 in 293 T cells (Deng et al., 2009). Shuai et al. synthesized biodegradable, lower cytotoxicity, superior buffering capacity and long circulation ternary copolymer carriers (PPI) by grafting low molecular weight linear PEI onto the block copolymer PLL–PEG, which could combine the PEI and PLL's advantages and minimize the side effects (Dai et al., 2011a). Transfection data indicated that 57% of linear PEI in PPI could remarkably improve the vector's ability for efficient gene transfection (Fig. 11). These studies demonstrated that balance between transfection efficiency and toxicity could be achieved by rational control of the structures of the PEI.

3.3. Poly(β-amino esters) (PBAEs)

Poly(β-amino esters) (PBAEs), which formed by the conjugation of amines and diacrylates (Fig. 12), are promising carriers for gene delivery due to their ability to condense plasmid DNA into small and stable nanoparticles and their ability to promote cellular uptake and endosomal escape (Green et al., 2008; Lynn and Langer, 2000; Martello et al., 2012; Shen et al., 2009). The PBAEs are pH-responsive, which could be soluble at an acidic pH but insoluble at the physiological condition. Anderson's lab had studied a combinatorial library of over 2000 structurally PBAEs, and found that diamine end-modified PBAEs (C32), prepared by conjugating 1,4-butanediol diacrylate with 5-amino-1-pentanol, exhibited higher effectivity for gene delivery than commercially available nonviral vectors including jet-PEI and Lipofectamine 2000 *in vitro* and *in vivo* (Fig. 12) (Green et al., 2007; Zugates et al., 2007). The end-modification therapy provides a useful method to develop and optimize cationic polymers for gene therapy. PBAEs with a reducible cystamine unit could dramatically improve siRNA delivery by facilitating quick release, causing up to 85% knockdown in primary human glioblastoma cells (Zhang et al., 2013).

Considering the influence of the linear and branched structures on the polymers' cellular uptake behavior and transfection efficiency, Shen et al. explored three bioreducible poly(amido amine)s (PAAs) to investigate the effect of different degrees of branching on endocytosis and gene transfection (Zhang et al., 2013). These poly(amido amine)s had the similar repeating units and molecular weights but with different degrees of branching. To increase the DNA release and reduce the toxicity, all three polymers were designed with disulfide linkages in the backbone for redox-cleaving in the condition of intracellular glutathione. In comparison with linear PAA, low-branched PAA polyplexes exhibited much higher transfection efficiency at the polymer/DNA ratios from 25 to 100, which were comparable with that of LPEI 25 k, and much less toxic than LPEI 25k against OVCAR-3 cells (Fig. 13). The structures of the low-branched PAA helped more polyplexes enter into cell membranes at a low polymer/DNA ratio than high-branched PAA, resulting in higher transfection efficiency in OVCAR-3 cells.

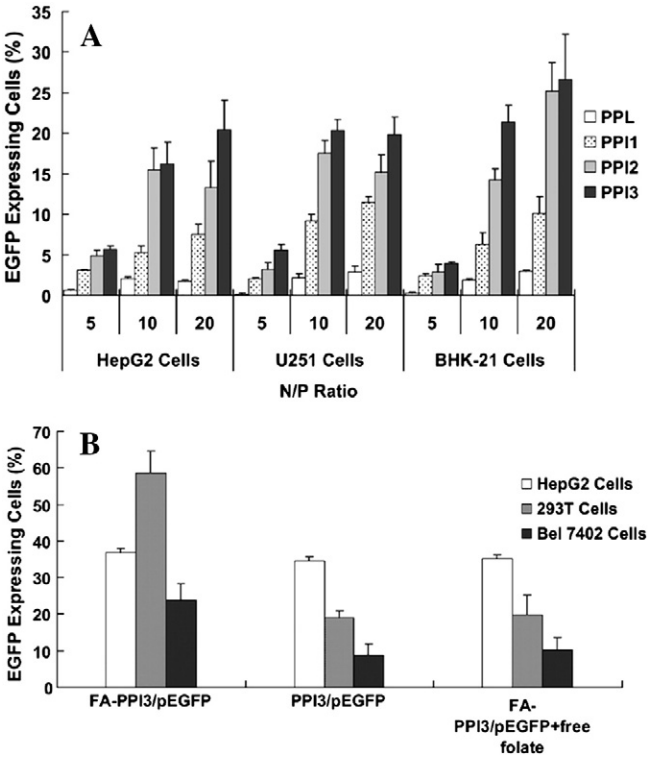

Fig. 11. (A) Transfection efficiency of PPL(PEG_{45}-b-PLL_{62})/pEGFP, PPI1(PEG_{45}-b-PLL_{62}-g-$(IPEI_{15})_{3.6}$)/pEGFP, PPI2(PEG_{45}-b-PLL_{62}-g-$(IPEI_{15})_{6.3}$)/pEGFP and PPI3(PEG_{45}-b-PLL_{62}-g-$(IPEI_{51})_{5.9}$)/pEGFP complexes determined by flow cytometry in HepG2 cells, U251 cells, and BHK-21 cells at various N/P ratios. Transfection was performed at a dose of 2 μg of DNA (mean ± SD, n = 3); (B) Transfection efficiency of FA-PPI3/pEGFP, PPI3/pEGFP, and folate competing assay determined by flow cytometry in 293 T, Bel 7402 and HepG2 cells at N/P 10. Reproduced with permission from Dai et al. (2011).

3.4. Lipid/polymer hybrid system

To marry the advantages of delivery systems based on lipid and polymer, cationic lipid, N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide (BHEM-Chol), was cooperated with the amphiphilic block copolymer poly(ethylene glycol)-b-poly(D,L-lactide) (mPEG–PLA) for siRNA delivery using a double-emulsion solvent evaporation technique by Wang et al. (Yang et al., 2011). This formulation could achieve high siRNA encapsulation efficiency, above 90%, with the aid of cationic lipid; and they could be effectively internalized by cancer cells and escaped from the endosome. Moreover, the systems carrying siRNA targeting the Plk1 gene were found to specifically downregulate expression of the oncogene Plk1 and induce remarkable apoptosis in HepG2 and MDA-MB-435s cancer cells following systemic administration. The systems ($NP_{0.2/1.0/25.0}$) carrying 200 nM of siPlk1 and above could achieve a comparable gene silencing level to that of Lipofectamine 2000 with 50 nM of siPlk1 (Fig. 14). Furthermore, the optimized lipid/polymer hybrid system based on PEG–PLA/PLA for delivering siPlk1 was able to inhibit tumor growth at 65% reduction in a BT474 xenograft murine model by silencing the Plk1 gene in tumor cells (Fig. 15) (Yang et al., 2012).

Poly(ethylene glycol) (PEG) is introduced into the liposomes and polymersomes for a long circulation time, which leads to efficient drug accumulation in tumors (Choi et al., 1998; Huang et al., 2010; Männistö et al., 2002; Namgung et al., 2009). Though PEGylation has gained great success in delivering chemotherapeutic drugs to tumors, it hampers *in vivo* applications for delivery of DNA and siRNA as PEGylation inhibits the interaction of the gene carriers with tumor cell surface, which results in poor cellular uptake and inefficient endosomal escape (Mintzer and Simanek, 2009). Studies also have shown that the

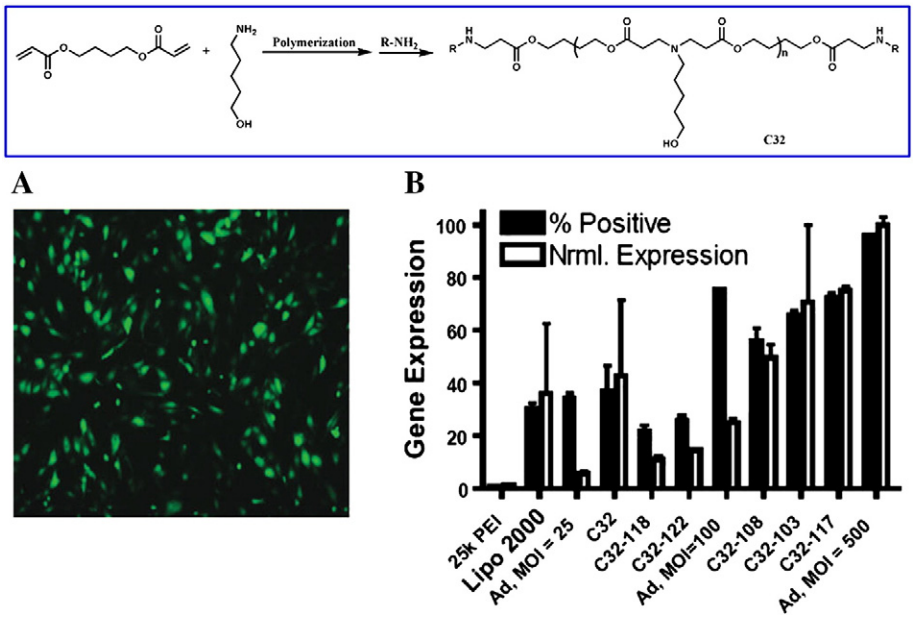

Fig. 12. The synthetic route of the diamine end-modified PBAE, C32 (upper). Gene expression of leading nonviral polymeric vectors compared with adenovirus vectors (lower): (A) fluorescent micrograph of GFP gene delivery mediated by polymer C32-103 at 24 h post-transfection; (B) gene expression compared on the basis of both the percentage of cells positively transfected and the normalized total gene expression per cell at 48 h post-transfection. Reproduced with permission from Green et al. (2007). Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

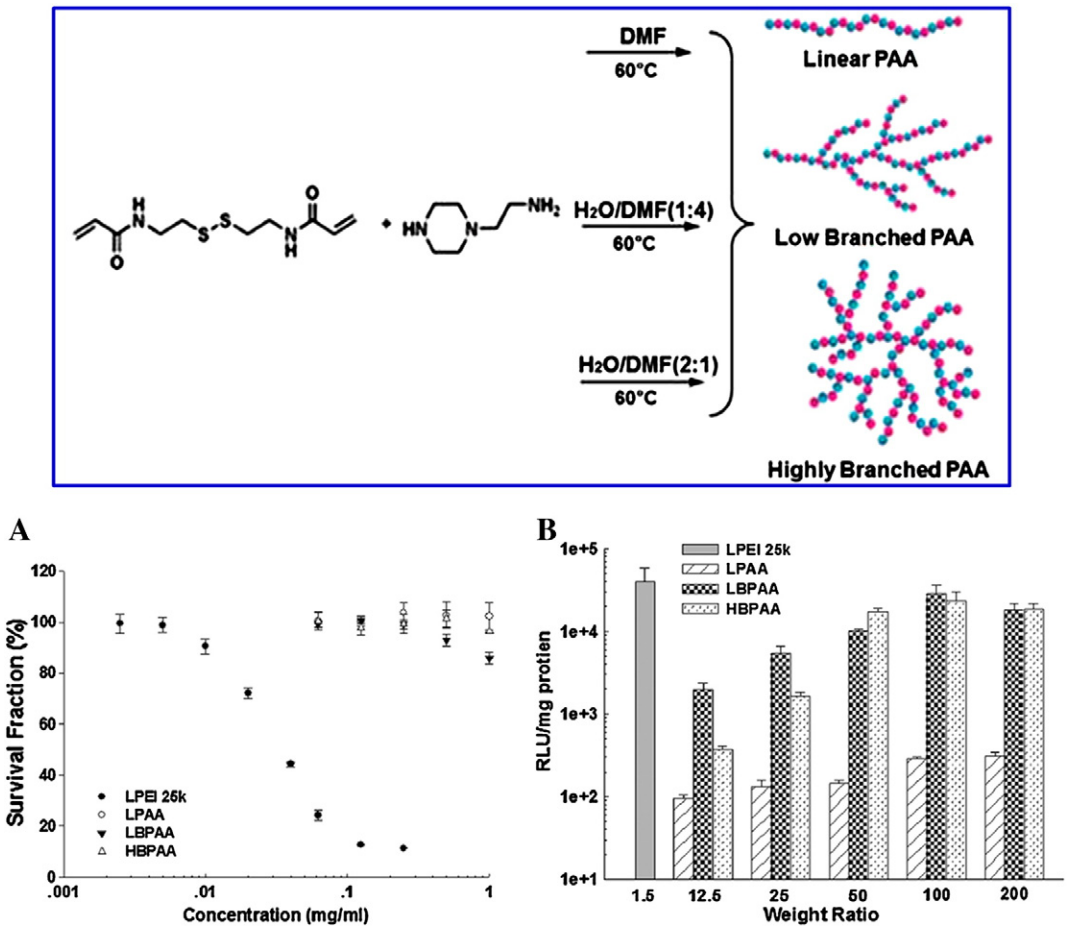

Fig. 13. Schematic illustration of the molecular structures of PAAs (upper). The cytotoxicity of PAAs and LPEI 25 k on OVCAR-3 cells at various polymer concentrations (lower A). Cells were treated with polymers for 4 h and cultured in fresh medium for another 44 h before MTT assay. The transfection efficiencies of PAAs and LPEI 25 k on OVCAR-3 cells (lower B). Cells were treated with polyplexes at different polymer/DNA ratios (pDNA dose of 10 mg/mL) for 4 h. The polyplex solution was then replaced with fresh medium and cultured for another 44 h before luciferase measurements. Reproduced with permission from Zhang et al. (2013). Copyright 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

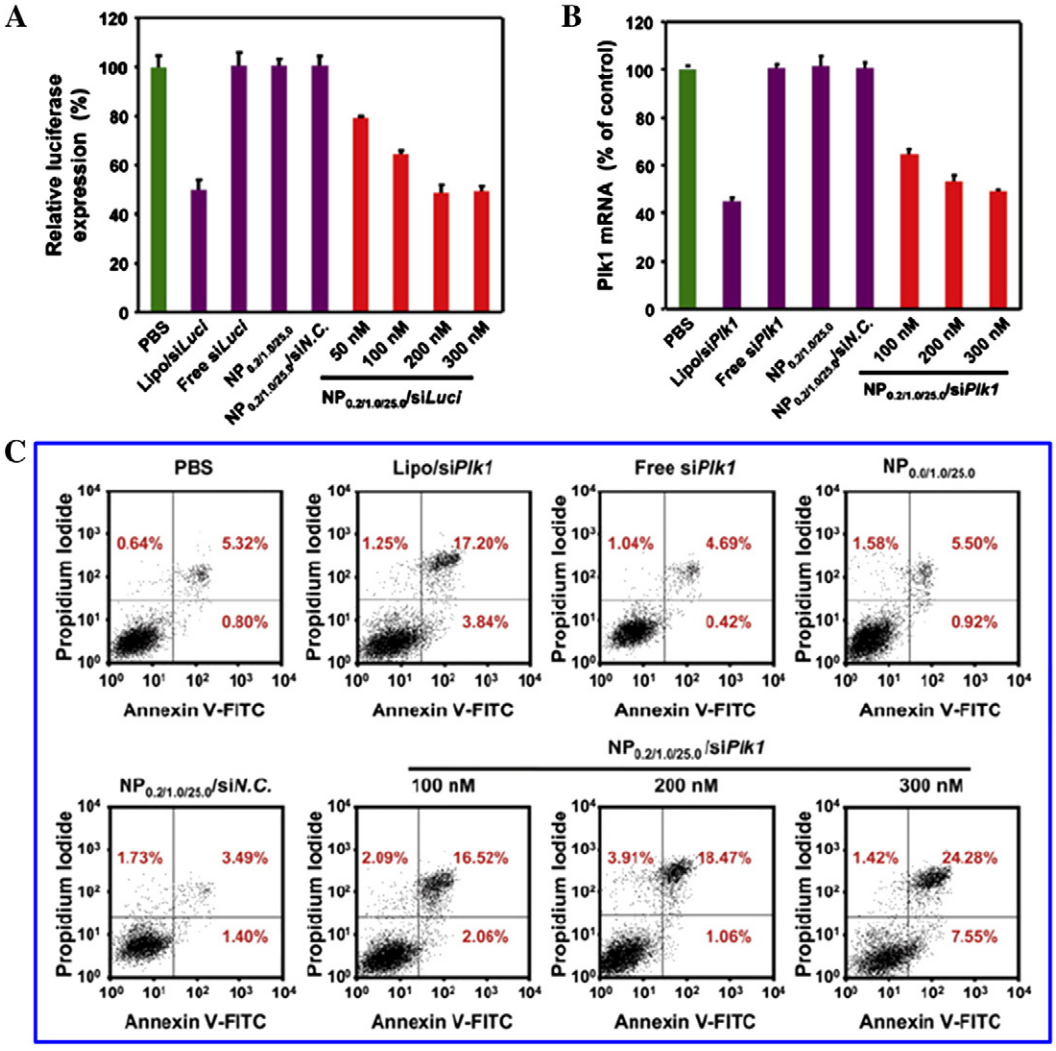

Fig. 14. (A) Relative luciferase expression in HepG2-luciferase cells after treatment with different formulations; (B) Plk1 mRNA expression in HepG2 cells determined by quantitative RT-PCR analysis following treatment with different formulations. (C) The influence of siPlk1 delivery by nanoparticles on apoptosis in HepG2 cells. Apoptosis was evaluated after treating HepG2 cells for 48 h. Early apoptotic cells are presented in the lower right quadrant and late apoptotic cells are presented in the upper right quadrant. Lipo/siLuci and Lipo/siPlk1 represent the complexes of Lipofectamine 2000 with siLuci (50 nM) and siPlk1 (50 nM), respectively. Free siLuci and free siPlk1 represent that cells were incubated with siLuci and siPlk1 at a dose of 300 nM, respectively. $NP_{0.2/1.0/25.0}$ and $NP_{0.2/1.0/25.0}$/siN.C. represent that cells were administered empty nanoparticles ($NP_{0.2/1.0/25.0}$, 0.549 mg/mL of polymer) and nanoparticles encapsulating siN.C. ($NP_{0.2/1.0/25.0}$/siN.C., 300 nM siRNA and 0.549 mg/mL of polymer), respectively. Reproduced with permission from Yang et al. (2011).

systems will emerge new resistance to PEGylation delivery vectors (Armstrong et al., 2007; Ishida et al., 2003). All of these name the "PEG dilemma". In order to overcome the shortcoming of the PEGylation, smart delivery vectors are created for successful gene delivery.

Harashima et al. developed a multifunctional envelop-type nano-device (MEND) for non-viral gene delivery to realize "programmed packaging" for overcoming the PEG dilemma. The MEND was prepared by using a lipid film hydration method, which consisted a nucleic acid core complexed with polylysine (PLL) and a lipid envelope equipped with multifunctional devices (Fig. 16). It exhibited increased cellular uptake and showed 1000-fold enhanced transfection activity than the DNA/PLL complexes *in vitro* (Kogure et al., 2004). By equipment with cleavage PEG moiety, the stable MEND performed an efficient tumor accumulation and *in vivo* transfection activity in comparison with the PEG-unmodified system (Hatakeyama et al., 2007). In 2012, they further designed a pH-sensitive cationic lipid, YSK05, to overcome the limitations of PEGylation. The YSK05-MEND induced efficient gene silencing compared with conventional cationic lipid formulated with DOTAP or DODAP both *in vitro* and *in vivo* due to effective endosomal escape (Fig. 17) (Sakurai et al., 2013; Sato et al., 2012).

4. Dendrimersome

Polymersomes as the nucleic acid delivery carriers still have the probability to dissociate in the complex environment *in vivo*. The appearance of dendrimers convinced the stability of formed self-assembled nanostructures. The first dendrimers were synthesized by Vögtle et al. in 1978 (Buhleier et al., 1978). It typically had a core and was symmetrical around with branched molecules, which formed a spherical three-dimensional morphology. In 2010, Percec et al. presented libraries of asymmetric structural denderimers (Percec et al., 2010). Prepared by rationally designed hydrophilic and hydrophobic branched parts, amphiphilic Janus dendrimers could facilitate self-assembly to various stable bilayer nanostructures that serve as delivery vectors for drugs, genes and so on (Fig. 18) (Caminade et al., 2012). They combined the mechanical strength from polymersomes and biological function from stabilized phospholipid liposomes, and exhibited superior uniformity of size, ease of formation and chemical functionalization; except for their complicated synthesis routes and difficult purified procedures. They were excellent candidates for models of biological membranes due to their equivalent membrane thickness to natural

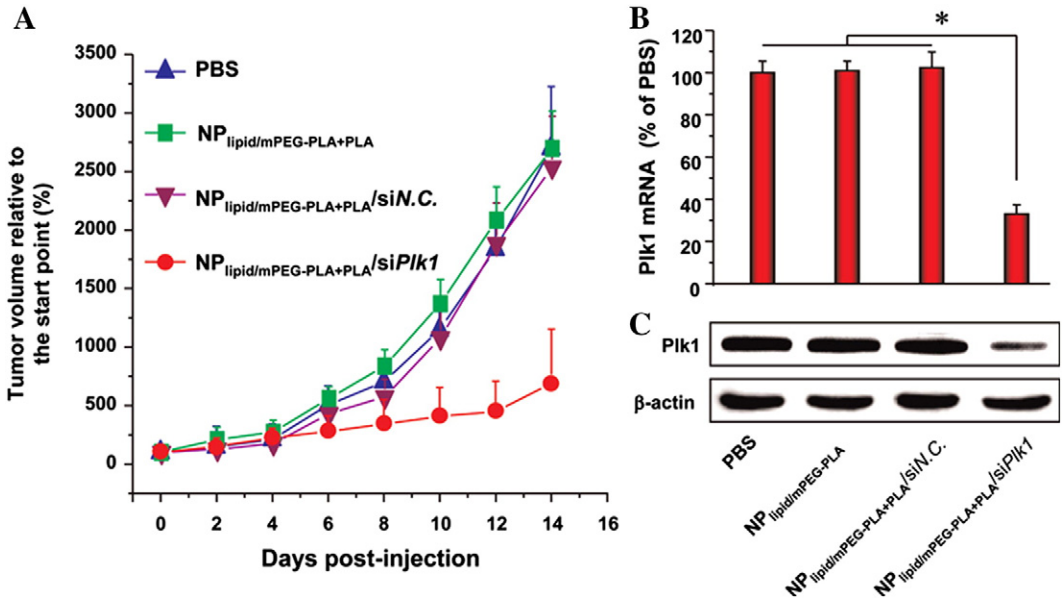

Fig. 15. (A) Inhibition of tumor growth in a murine model with BT474 xenografts after treatment with various formulations (n = 6). Expression of Plk1 mRNA (B) and protein (C) in tumors was analyzed 24 h after the final injection. BT474 xenograft tumor-bearing mice received one intravenous injection everyday from the 10th day post-xenograft implantation in all of the experiments. $NP_{lipid/mPEG-PLA + PLA}$, $NP_{lipid/mPEG-PLA + PLA}$/siN.C., and $NP_{lipid/mPEG-PLA + PLA}$/siPlk1 represent that the mice were administered with empty hybrid nanoparticles $NP_{lipid/mPEG-PLA + PLA}$, $NP_{lipid/mPEG-PLA + PLA}$ carrying siN.C., and siPlk1 at the same weight of hybrid nanoparticles (3.05 mg per injection), respectively. The N:P ratios were all 10:1 in the experiments, and the dose of siRNA was 10 μg per mouse per injection; * $p < 0.005$ (n = 6). Days after the first injection. Reproduced with permission from Yang et al. (2012). Copyright 2012 American Chemical Society.

bilayer membranes distinct impermeability and mechanical properties (Peterca et al., 2011).

From the mechanical properties of denderimersome, they stay quite stable, easy to construct vesicles of uniform size, which make them an excellent carrier for application of delivery drugs, nucleic acids and many other substances. However, it will still take time for the dendrimersome to be well constructed in large scale, and the complicated preparation methods for Janus denderimers need to intelligently develop. Until now, there has no information reported about the denderimersome for nucleic acid delivery maybe due to their specific requirements.

5. Summary and perspective

Nucleic acids have inherited drawbacks in delivering into targeted tissues and cells. To overcome these obstacles, a large number of delivery systems have been reported. They have shown promising efficacy in gene transfection and gene silencing and the treatment of various diseases. Mimicking from virus vectors, cationic lipids are the first materials used for nucleic acid delivery. They are generally used with co-lipids to form small sizes of liposomes. The transfection efficiency of lipoplexes was influenced by the structures and charge density of the cationic lipid, the lipid/nucleic acid ratio, the charge and size of the lipoplex and so on. However, the low gene transfection efficiency and instability in the physiological condition result in unimpressive data in clinical trials. Cationic polymers always contain a number of amine groups. They can be easily prepared and multifunctionalized for specific requirements, whereas toxicity and efficient transfection *in vivo* are the bottlenecks for cationic polymers stepping towards clinical use. However, Wu and Andresen et al.'s recent results confirmed that free cationic polymers in the solution mixture of DNA and polymers helped to promote gene transfection through faster cellular internalization and intracellular trafficking of the polyplexes (Dai et al., 2011b; Yue et al., 2011a, 2011b), and focused on discussing the intracellular trafficking mechanisms of cationic polymers and DNA (Yue and Wu, 2013), which brought new dawn to us for the

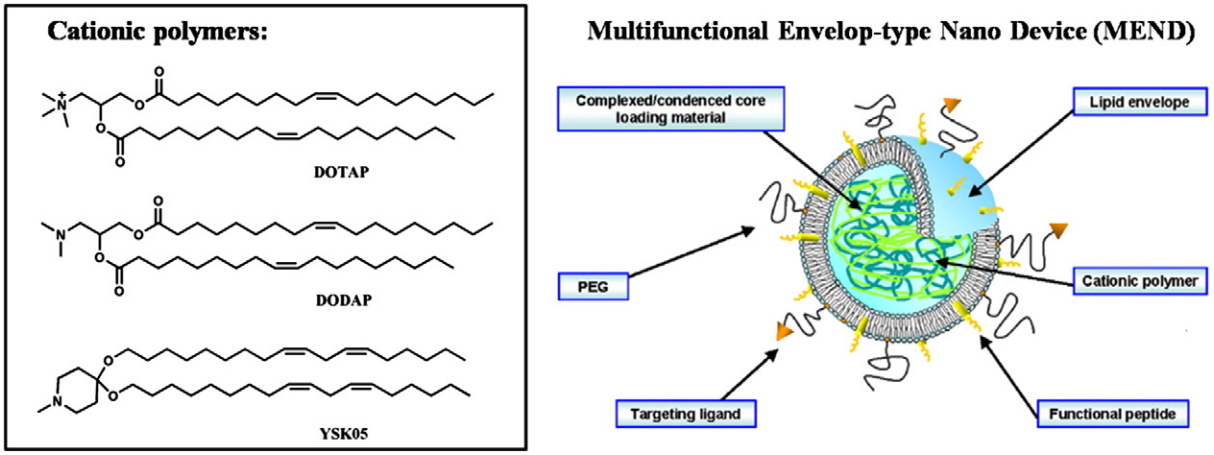

Fig. 16. Schematic representation of a multifunctional envelop-type nano-device (MEND). The MEND consists of a complex nucleic acid core, coated with a lipid envelop modified with functional devices. Reproduced with permission from Hatakeyama et al. (2011).

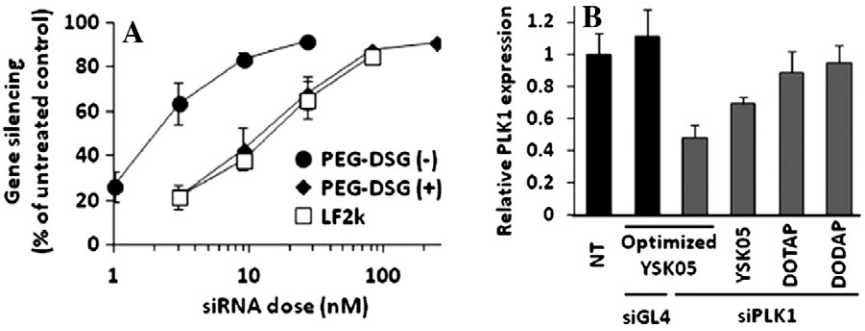

Fig. 17. Gene silencing activity of PEGylation optimized YSK05-MEND in vitro and in vivo. (A) MEND mediated gene silencing *in vitro*. HeLa-luc cells were treated with MENDs and Lipofectamine 2000 (LF2k) incorporating anti-luciferase siRNA (siGL4) for 24 h (n = 3). (B) MEND mediated gene silencing *in vivo*. OS-RC-2 tumor bearing mice were topically administrated with MENDs at a dose of 10 μg siPLK1 or siGL4 into tumors. After 24 h, the mice were euthanized and PLK1 gene expression was measured (n = 4). Reproduced with permission from Sato et al. (2012).

development of non-viral carriers. Marrying advantages of these two delivery vectors, polymer/lipid hybrid systems might provide further improvement for safety and efficient delivery.

Except for these, deep and comprehensive understanding of the obstacles in the delivery to cells will guide us to intelligently design smart biomimic vectors. The route of nucleic acid vectors entering the cells and performing their functions is a complicated molecular biological process. It contains circulation in physiological condition, entering cell membranes through endocytosis, escaping from the endosomes, unpacking of the complexes of nucleic acids and release of the intact nucleic acids in the cytoplasm for siRNA or in the nucleus for DNA. Especially for delivery of DNA, how DNA pass through the nuclear membranes, which is not only count on the mitosis state of cells, still need us for better and comprehensive understanding of the process of the cell biology. Combination of the multidisciplinary comprehension will help us to construct rational multifunctional vectors for gene therapy to overcome or avoid the hampers set up by cells.

Another potential way to further improve gene therapy is to combine gene therapy with stem-cell therapy. Stem cells have the ability to self-renew and differentiate. Introduction of stem cells into damaged tissues has the potential to cure diseases and replace the defections. Recently, stem cells are widely studied for their potential therapeutic use. They play an important role in the progress of the diseases, including cancers. For example, the most current anticancer therapies are to kill cancer cells, and they always can't obtain long-term ideal promise. Cancer stem cells are believed to be the target cells responsible for malignant transformation, and the tumor formation may be due to the disorder of stem cells (Dean et al., 2005). Therefore, effective anticancer treatments could be achieved in targeting and destroying the cancer stem cells (Li et al., 2013). However, there have been other normal stem cells, comprehensive understanding and identification of the various forms of cancer stem cells and performing gene identification methods could help to determine the differences from normal stem cells and cancer stem cells, and further to aid in the development of effective treatments to cancers (Soltanian and Matin, 2011). Herein, proper targeting for the non-viral vectors should be very important for efficient gene therapy, which needs us get a close connection with biology. And combining the gene therapy with stem cells and collaborating with multidisciplinary knowledge will provide a new approach for disease therapy and guide us for rational design of smart non-viral vectors for gene delivery, and maybe the ultimately route for disease treatments.

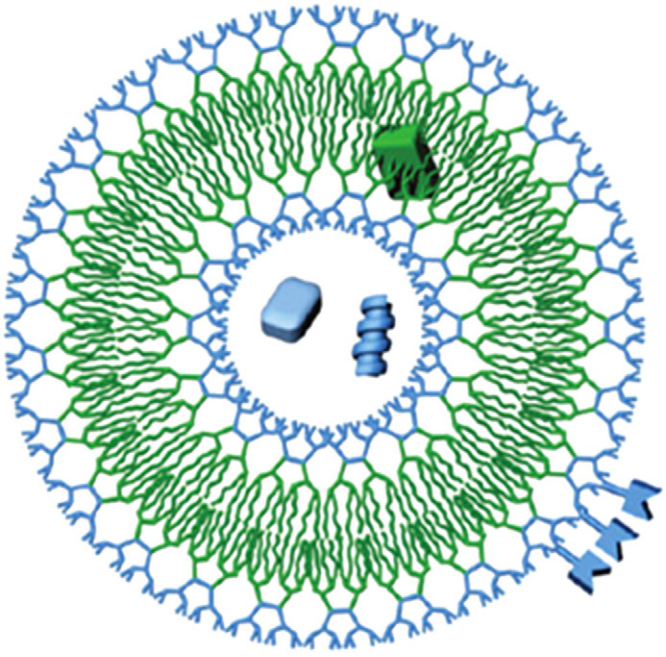

Fig. 18. Denderimersomes assembled from amphiphilic Janus denderimers. The color code used is: green for the hydrophobic parts and blue for the hydrophilic parts. Adapted with permission from Peterca et al. (2011). Copyright 2011 American Chemical Society.

Acknowledgments

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