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Review

Non-viral gene delivery carrier and its three-dimensional transfection system

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ABSTRACT

An increasing number of non-viral vectors are being developed for the use of gene delivery nowadays, among which cationic polymers and lipoplexes receive most attention. Most of these researches are focused on how to increase the transfection efficiency of non-viral vectors as well as the reduction of toxicity. In this review, we go over new strategies to reduce the toxicity of cationic polyplexes such as poly(ethylene-imine) and the construction of highly effective gene transfer vector lipoplexes. In addition, since transformation of gene expression system from two-dimensional (2D) substrate to 3D scaffold triggers far better transfection efficiency, the non-viral vectors applied in 3D transfection system have also been reviewed.

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1. Introduction

The basic concept underlying gene therapy is that human disease may be treated by the transfer of genetic material into specific cells of a patient in order to enhance gene expression or to inhibit the production of a target protein (Kawakami et al., 2008). One of the bottleneck that must be overcome in order to bring more gene therapies to the clinic is the development of efficient gene delivery agents, vectors. To realize gene therapy, an intelligent vector that satisfies the requirements for both a high transfection activity and poor cytotoxicity is essential (Hama et al., 2007). In theory, viral carriers could provide a high transfection rate and a rapid transcription of the foreign material inserted in the viral genome. However, many clinical trials in which viral vectors are used have been interrupted since the application of these vectors induced unexpected adverse effects such as immunogenicity and oncogenicity (Marshall, 1999; Hacein-Bey-Abina et al., 2003). Non-viral vectors have several advantages such as ease of synthesis, cell/tissue targeting, low immune response, and unrestricted plasmid size (Cho et al., 2001; Nakamura et al., 2006). The most severe bottleneck in the clinical use of a non-viral vector is its low transfection activity. Therefore, an improvement of its transfection activity is required. So far, a variety of non-viral delivery approaches have been developed, including calcium phosphate (Fu et al., 2005; Olton et al., 2007), cationized liposomes (Dass and Choong, 2006; Li et al., 2008a), and noisomes (Huang et al., 2008).

On the other hand, transfection system is also demonstrated it is an indispensable part for efficient gene delivery and gene expression (Fig. 1). For example, transfection system can greatly affect the growth condition of cells, the sensitivity of the carriers to gene uptake as well as the condition of carriers in serum. Up to now, reverse transfection approach, which is different from the conventional approach and has been demonstrated that it could protect the carriers from the influence of serum, or combined the reverse method with biodegradable or non-biodegradable three-dimensional (3D) scaffolds have been reported (Hosseinkhani et al., 2006; Okazaki et al., 2007). Therefore, the novel transfection systems used for enhancing the efficiency of gene carriers are also summarized in this review.

2. Polymer-based gene delivery system

Among the non-viral vectors, cationic polymers have gained increasing attention because they can easily form polyelecrolyte complexes between plasmid DNA and cationic polymers, and also, mediate transfection via condensing DNA into nanoparticles, protect DNA from enzymatic degradation, as well as facilitate the cell uptake and endolysosomal escape (Park et al., 2005). Frequently studied cationic polymers include poly(ethylene-imine), poly(L-lysine) (PLL), chitosan and dendrimers.

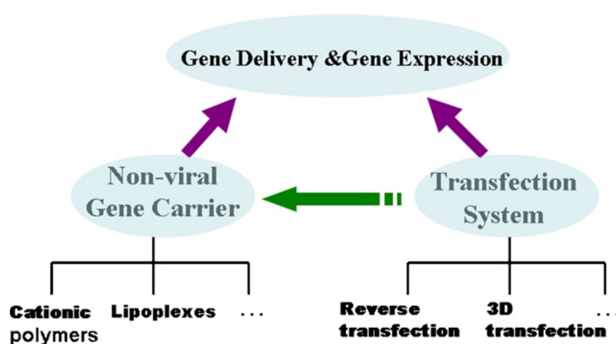

Fig. 1. The efficiency of gene delivery is dependent on both gene carriers and transfection system. A suitable transfection system can promote the application of non-viral gene carries.

PLL are linear polypeptides and thus posses a biodegradable nature. However, PLL has poor transfection ability when applied alone or without modifications (Farrell et al., 2007; Männistö et al., 2002). The relatively low transfection level of PLL is possibly due to inadequate escape of the complexes from endosomes or the inefficient release of DNA from the complexes (Midoux and Monsigny, 1999). In order to increase the transfection ability of PLL, histidine residues have been introduced to the backbone of PLL to provide the endosome escape property (Midoux and Monsigny, 1999). Recently, conjugating PLL with chitosan or lipids such as palmitic, myristic and stearic acids have also been reported to be able to increase the transfection efficiency of PLL (Yu et al., 2007; Abbasi et al., 2008). However, the mechanisms of the enhanced transfection ability of these PLL-based polymers have not been demonstrated.

Chitosan is another hotspot in the field of non-viral gene delivery system due to its good biocompatibility and a high positive charge density (Weecharangsan et al., 2008). Gene delivery efficiency of chitosan is affected by a lot of factors, including chitosan molecular weight, salt form, degree of deacetylation, the pH of the culture medium and so forth (Kim et al., 2007). Even with the optimized formulation, the application of chitosan-based gene delivery system is still limited by the low water solubility of chitosan, inefficient gene unpacking and therefore low gene transfection efficiency as compared with other cationic polymer. In recent years, various chitosan derivatives have been synthesized aiming to resolve these two problems and the structure modification of chitosan can be chiefly divided to hydrophilic modification and hydrophobic modification (Kim et al., 2007). Incorporation of negatively charged agents such as hyaluronic acid (HA) or poly(γ-glutamic acid) (γ-PGA) with chitosan has been shown to be able to increase transfection efficiency significantly (Duceppe and Tabrizian, 2009; Peng et al., 2009). Such improvement in the transfection ability of chitosan-based carrier could be attributed to the low charge density of the HA chain or the formation of γ-PGA/chitosan/DNA complexes which might disintegrate into a number of even smaller sub-particles after cellular internalization, both of which could improve the easy release of DNA. It merits a mention that the choice of the type and the amount of anionic polymer incorporated to chitosan/DNA complexes could greatly influence the transfection ability through the changes of cellular uptake, stability, size of the nanoparticles, condensing and dissociation ability with DNA (Duceppe and Tabrizian, 2009; Peng et al., 2009).

Dendrimers such as polyamidoamine (PAMAM) and polypropylenimine (PPI) have also been studied for gene delivery *in vitro* and *in vivo* for their high transfection efficiency (Dufès et al., 2005). However, the toxicity of dendrimers is of major concern for their medical use. Generally, *in vivo* toxicity of dendrimers is related with various factors including the chemical structure, surface charge, generation and dose of dendrimers (Aillon et al., 2009). Surface modification with PEG or replacement with low generation dendrimers have been reported to be able to improve the biocompatibility of these biomaterials (Russ et al., 2008; Jevprasesphant et al., 2003).

As PEI is one of the most widely studied non-viral vector due to its high transfection ability, and much efforts has been made to decrease its toxicity, we mainly focus on the current progress of PEI and its derivatives as the gene carrier. In addition, the biodegradable polymers, which have gained increasing attention during the past decade for their reduced toxicity and prevention of polymer accumulation in the body, are also discussed.

2.1. PEI-based gene delivery

Poly(ethylene-imine) has been revealed to be the most effective non-viral vector based on favorable characteristics of DNA

protection, cell binding and uptake, endosomal escape and release from the carrier (Zhang et al., 2004). PEI of a certain high molecular weight is necessary for efficient delivery of DNA, however, a high molecular weight PEI is cytotoxic, and its long-term safety is problematic because of its nonbiodegradability (Kircheis et al., 2001). Thus, to increase the transfection efficiency of PEI-based polyplexes and reduce its cytotoxicity, various strategies have been formulated.

2.1.1. PEI-chitosan

Chitosan is considered to be a good candidate for a gene delivery carrier with its well biocompatibility, biodegradability, low toxicity and high cationic potential. However, this material has low transfection efficiency (Lee et al., 1998). In our previous study, PEI's cytotoxicity was considerably decreased upon simply incubation with the negatively charged chitosan/DNA complex through static electronic interaction. Furthermore, the transfection efficiency of the PEI/chitosan/DNA complex increased to 1000-fold of that induced using chitosan alone and almost the same as that induced using PEI (M_w = 25k) (Zhao et al., 2008).

However, it is difficult to elucidate the way how PEI interacts with chitosan during the formation of complex only via incubation and to control the structures of the products such as the molecular weights and constitutions. Therefore, we synthesized chitosan-graft-polyethyleneimine (CHI-g-PEI) copolymer by an imine reaction between chitosan and low molecular PEI. The CHI-g-PEI copolymer had maintained low cytotoxicity as that of the chitosan/DNA complex, and showed a higher *in vitro* transfection efficiency than PEI (M_w = 25k) in HeLa, A549 and HepG2 cell lines at high N/P ratio (data unpublished). Furthermore, a polypeptide binding to HepG2 screened by biopanning phage display random heptapeptide library was conjugated with CHI-g-PEI, and *in vitro* results showed that peptide- conjugated CHI-g-PEI did have a specificity for HepG2 rather than human normal hepatic cells (L02 cells), suggesting the potentially limited adverse effect of this novel vector (data unpublished).

Wong et al. synthesized polyethyleneimine-graft-chitosan (PEI-g-chitosan) by performing cationic polymerization of aziridine in the presence of oligo-chitosan (M_w = 3400) (Wong et al., 2006). The *in vivo* transfection efficiency of the complexes of PEI-g-chitosan/DNA was evaluated through administration in common bile duct in rat liver. The results indicated that the complex of PEI-g-chitosan/DNA showed the highest transfection efficiency when the N/P ratio was 10:1. In contrast, the optimal N/P ratio for *in vitro* transfection was 40:1. The author explained that the discrepancy may be because more stable complexes of PEI-g-chitosan/DNA are needed for efficient uptake by cells *in vitro* as compared to the *in vivo* transfection when a more concentrated suspension of the complexes was injected directly to the common bile duct. Probably the weaker association of DNA and the low molecular weight PEI-g-chitosan would result in the ease of DNA release from the complexes, which would facilitate the transfection of DNA.

2.1.2. PEI-ligand

As mentioned above, the presence of positive charges at the surface of DNA complexes promotes non-specific interactions with plasma proteins and cell membrane proteins. To overcome this problem, the most attractive strategy is to replace the non-specific electrostatic interactions between cells and the transfection complexes with a cell-specific interaction that triggers receptor-mediated endocytosis of the DNA complexes (Kichler, 2004).

Integrin-binding arginine–glycine–aspartic acid (RGD) ligand is widely used as targeting ligand. Our previous study demonstrated that modified the adenovirus vector in fiber site with RGD enhanced the gene transfection efficiency notably in CAR-deficient cells (Gao et al., 2004). When used in non-viral vectors, RGD ligand often chemically attached to polycation vector via hydrophilic polyethylene glycol (PEG) spacer. Coating of the PEI/DNA complexes by PEG-Suc which bears 17.7 pairs of carboxylic acid-side chains recharged their surface to be negative, and effectively protected them from the albumin-induced aggregation (Sakae et al., 2008). RGD-PEG-Suc-coated plasmid/PEI complex brought about more than 3 times higher reporter protein activity on the cultured B16 cells. Those bio-compatible DNA complexes with ligand attained very high gene expression level in tumor, lung, and liver after injection into mouse tail vein.

2.1.3. Low molecular PEI

Several groups have reported the potential cytotoxicity against various cell lines of high molecular weight PEIs such as PEI (M_w = 25k) (Fischer et al., 1999). Also, the high affinity of PEI (M_w = 25k) for DNA is another important barrier of cytosolic delivery which would limit the overall transfection efficiency due to the relatively inefficient dissociation of pDNA from PEI, whereas low molecular weight (LMW) PEI is less toxic but shows almost no transfection (Godbey et al., 1999). Therefore, modification of low molecular PEIs has been studied extensively to improve gene transfer efficiency while keeping cytotoxicity manageable (Choi and Lee, 2008). Several investigators synthesized highly branched polymers consisting of LMW PEI and degradable cross-links. Cross-linkers include PEG-bis-succinimidyl succinate (Ahn et al., 2002), glutaraldehyde (Kim et al., 2005), disulfide-containing agents such as dithiobis (succinimidyl propionate) (DSP) and dimethyl-3, 3′-dithiobispropionimidate (DTBP), etc. (Gosselin et al., 2001).

Xu et al. cross-linked LMW PEI (M_w = 0.8k) with 1, 4-butanediol bis (chloroformate) to form a novel PEI biscarbamate conjugate (PEIC) with a low molecular weight (M_w = 2800, M_n = 910) (Xu et al., 2008). PEIC showed lower cytotoxicity and orders of magnitude higher luciferase gene transfection activity compared with PEI (M_w = 25k). In another approach, degradable poly(ester amines) (PEAs) based on glycerol dimethacrylate (GDM) and LMW PEI were synthesized (Arote et al., 2008). The PEAs showed significant lower cytotoxicity in three different cells (HeLa, HepG2 and 293T cells) compared with PEI (M_w = 25k) and has a much higher transfection efficiency than conventional PEI (M_w = 25k) and Lipofectamine. It was hypothesized that the higher transfection efficiency of PEAs was the synergistic effect arising from hyperosmotic glycerol and endosomal buffering capacity of PEAs which result from the presence of a glycerol backbone and PEI amine groups respectively.

2.1.4. In vivo behavior

Although polymer-based gene delivery could be modulated to reach a high transgene level, the discrepancy between *in vitro* study and *in vivo* behavior has been increasingly emphasized by more and more groups (Grosse et al., 2008; Burke and Pun, 2008; de Wolf et al., 2007). Recently, pharmacokinetics, biodistribution and tissue gene expression profiles as well as toxicity of polyplexes were investigated. Since the cationic nature of polyplexes tends to induce the aggregation and cleavage by macrophage system, polymers like PEI are always PEGylation to shield the positive charge (Merdan et al., 2005; Ogris et al., 2003). The *in vivo* behavior of PEG-PEI is reported to be dependent on the amount of pDNA loaded and the surface modification of the polymer (Merdan et al., 2005). At a low dose of pDNA, PEG-PEI/DNA complexes showed a greatly enhanced circulation time in the bloodstream, while at a higher dose, PEG-PEI/DNA complexes exhibited similar distribution profiles as PEI/DNA complexes did. In *in vivo* gene expression studies, luciferase expression could only be detected at the high dose of pDNA in the PEI/pDNA complexes. However, significant levels of the reporter gene were detected in lung, liver and spleen, which may cause severe acute toxicity (Merdan et al., 2005). It was

reported by Grosse that the intracellular trafficking of lactosylated PEI/DNA complexes through nasal instillation was not the same case as observed in the *in vitro* model. In *in vitro* study, it was usually reported that in airway epithelial cells, PEI/DNA entered the nucleus mostly as a complex. On the contrary, intracellular dissociation between the DNA and the vector was always observed in *in vivo* experiments where plasmid was mostly localized in lysosomes while the Lac-PEI localized in the nucleus (Grosse et al., 2008).

There are also a number of reports of the specific delivery to target the tumor site through systemic delivery of PEI-based polymer, the toxicity of which vary from case to case or have not been precisely investigated (Liang et al., 2009; Pathak et al., 2009). PEG could protect the polyplexes from being cleared during the circulation, but may hinder subsequent efficient intracellular activity of gene. In order to overcome this dilemma, a kind of pH-reversible polymer PEI-mPEG-HZN-NHS has been synthesized (Fella et al., 2008). PEG was presented in the polyplexes and would be removed from the polyplexes at endosomal pH. The luciferase activity was highest in the tumor tissue and only a very low transgene expression was observed in liver and lung after intravenous administration in a subcutaneous HUH7 tumor model in SCID mice. In another study, after intravenous administration of spherical M-PEIs/pDNA nanogels into tumor bearing nude mice, the GFP expression is predominantly found in the tumor tissues and hardly detected in the surrounding nontumor liver tissues and lung, and undetectable in other major organs (Dong et al., 2009), indicating PEI-based carrier would be a efficient gene delivery system for the tumor therapy.

2.2. Biodegradable polymers

The backbone linkages of most polymeric gene carriers consist of a carbon–carbon bond or amide bond, which are not degraded in physiological solutions. The non-degradable non-viral carriers are not easily removed by physiological clearance systems and therefore can possibly accumulate within cells or tissues to elicit further cytotoxicity. To address the problems, several biodegradable polycations with much less cytotoxicity as compared with an unmodified polycations, such as PLL or PEI, have been synthesized and evaluated as potential gene carriers (Park et al., 2006).

Biodegradable poly(2-aminoethyl propylene phosphate) (PPE-EA) consists of a phosphoester backbone and an aminoethoxy side chain (Wang et al., 2001). The phosphoester backbone of PPE-EA could be hydrolyzed to generate propylene glycol, phosphate and ethanolamine in physiological condition. The polymer had significantly lower cytotoxicity at a concentration up to 0.1 mg/ml as compared with PEI and PLL. PPE-EA showed much higher transfection efficiency than PLL, especially in the presence of chloroquine (Wang et al., 2001).

Choi et al. synthesized poly(ester amine) (PEA) by conjugating PCL with PEI (M_w = 1.2k). At physiological pH, PEA was hydrolyzed to be molecular weight products with less toxic products due to the susceptible ester linkage with a degradation half life of five days. It was also shown that PEA/pGL3 complexes also successfully transfected into the HNE cells with higher viability of the cells (Choi et al., 2007).

The biodistribution and *in vivo* transfection efficiency of polyplexes composed of plasmid DNA and biodegradable polymer, poly(2-dimethylamino ethylamino) phosphazene (p(DMAEA)-ppz) were investigated after intravenous administration in tumor bearing mice. p(DMAEA)-ppz were rapidly cleared from the circulation and showed considerable accumulation in the liver and the lung and had a substantial tumor accumulation of 5% ID/g for p(DMAEA)-ppz polyplexes at 4 h after administration, resulting in considerable reporter gene expression (de Wolf et al., 2005).

3. Lipoplexes

Since cationic lipid/DNA complex (lipoplex) was firstly introduced by Felgner et al. (1987) who used *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trime-thylammonium chloride (DOTMA) to carry out a DNA-transfection protocol, cationic lipids and polycations used for DNA transfer have been widely investigated (Caracciolo et al., 2008; Tenchov et al., 2008). The structures of complexes are dependent on composition of the cationic lipids, neutral helper lipids, and the DNA. The main drawback of the application of these cationic lipids is their low transfection efficiency. Tremendous efforts are currently paid worldwide to elucidate this problem, including the continuous synthesis of new cationic lipids and new approach of complexes formulations.

3.1. Cationic liposomes

Cationic lipid-based liposomes have better biocompatibility and are quite effective for the delivery of DNA into the cytosol through endosomal pathway (Hideyoshi et al., 2001), while cationic polymers like PEI, can condense DNA efficiently and are more stable (Zhang et al., 2004). We designed the novel polycation liposomes (PCLs) constructed by using PEI (M_w = 800)-cholesterol (PEI 800-Chol), which combined the advantages of both cationic polymers and cationic lipids. PCLs showed a lower cytotoxicity and a higher transfection efficiency in HeLa cells, and there was no decrease of the transfection efficiency in the presence of 10% serum. These characteristics are favorable for the *in vivo* application of gene delivery systems. Furthermore, incorporation of dioleoylphosphatidylethanolamine (DOPE) could remarkably increase the transfection activity of PCLs (Chen et al., 2007). In the later report, PCLs also significantly enhanced the growth inhibition effects of antisense oligodeoxynucleotides (ASODN) against tumor cells, suggesting that PEI 800-Chol/DOPE liposomes would be a promising vector for ASODN delivery and improved antisense therapy (Chen et al., 2009).

In another research, cationic liposomes (CL) containing 3β [*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and DOPE, were constructed and used as carriers to facilitate the efficient transfer of ASODN into cells and to induce much better tumor-suppressive effect. The results showed that CL-ASODN complex could significantly enhance the inhibition efficiency of hTERT-ASODN in the growth of tumor cells. Human telomerase reverse transcriptase (hTERT) is a catalytic subunit of telomerase, which was reported to be over expressed on more than 90% of malignant tumor cells (Yang et al., 2002; Meeker and De Marzo, 2004). ASODNs alone did not have any tumor suppressive activity, the tumor-growth inhibitory rate of the CL-ASODN complex and 5-FU alone was 55.11% and 70.23%, respectively; however, the inhibitory rate increased to 89.47% when CL-ASODN was co-administrated with 5-FU. Our results suggested that CL containing DC-Chol and DOPE could be promising as a non-viral telomerase inhibitor in cancer antisense therapy (Han et al., 2008).

3.2. Lipid-coated DNA complexes

Cationic polymers are efficient gene delivery vectors in *in vitro* conditions, but these carriers may fail *in vivo* due to the interactions with extracellular polyanions. Accordingly, a neutral or negatively charged surface and the use of a target cell-specific ligand might be preferable to solve these problems. Lehtinen et al. (2008) developed lipid-coated DNA complexes (LCDC) that are resistant against the extracellular poly-anions, but become membrane fusogenic at acidic pH. The core of the complex is

DNA/PEI polyplex which is easily disintegrated by polyanions and capable of releasing DNA. Cationic DNA/PEI complexes were coated by DOPE and cholesteryl hemisuccinate (CHEMS) using two coating methods: detergent removal and mixing with liposomes prepared by ethanol injection. Only detergent removal produced lipid-coated DNA complexes that were stable against GAGs, but were membrane active at low pH towards endosome mimicking liposomes. The cell uptake of LCDCs was 1/5 compared to cationic polyplexes and the transfection efficiency *in vitro* was about 80% of the transfection mediated by uncoated PEI polyplexes the transfection efficiency of LCDCs was also decreased in the presence of serum, even though their cell uptake increased. It was inferred by the authors that the negatively charged lipid coat reduced the adhesion of the complexes on the cell surface and the loss of acid in DOPE induced fusogenity in the presence of serum.

In another study, an efficient non-viral gene transfer system named lipopolyplex has been developed by employing PEI, 1, 2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol as lipids at three different lipid/DNA molar ratios by using five different protocols of formulation (Garcia et al., 2007). In one of the protocols that could generate maximal transfection activity, DNA was condensed with PEI first and then be coated with lipids. At lipid/DNA molar ratio of 17:1 these lipopolyplexes showed high efficiency in gene delivery of DNA to liver cancer cells, even in the presence of high concentration of serum (60% FBS), much more efficient than conventional lipoplexes and polyplexes. The same behavior was observed for complexes prepared in the presence of the therapeutic gene pCMVIL-12. Toxicity assays revealed a viability higher than 80% in all cases, independent of the protocol, molar ratio (lipid/DNA), molecular weight and type of PEI (Han et al., 2008).

3.3. Niosomes

Synthetic surfactant vesicles, niosomes, a self-assembly of non-ionic amphiphiles, are widely studied as a substitute for liposomes because of their similar structures. Compared to their phospholipid liposome counterparts, niosomes offer many advantages for drug delivery. For instance, niosomes have been shown to have great physico-chemical stability; they can be stored up to 84 months without significant morphological changes (Uchegbu and Vyas, 1998). We previously reported the use of cationic niosomes as a potential gene carrier and showed that Span cationic niosomes exhibited positive results for gene delivery (Huang et al., 2005). However, positively charged particulates are prone to non-specific interactions with plasma proteins, which will lead to the destabilization, dissociation, and rapid clearance of gene/carrier complexes (Meyer et al., 1998). This greatly limited the application of cationic niosomes in biological fluid environment. Recently, polyethylene glycol (PEG) modified cationic niosomes were used to improve the stability and cellular delivery of oligonucleotides (OND) for their low cost and superior chemical and storage stabilities. Complexes of PEGylated cationic niosomes and OND showed a neutral zeta potential with particle size about 300 nm. PEG-modification significantly decreased the binding of serum protein and prevented particle aggregation in serum. The loaded nuclear acid drug exhibited increased resistance to serum nuclease. Compared with unmodified cationic niosomes, the PEGylated niosomes showed a higher efficiency of OND cellular uptake in serum. Therefore, because of their stable physiochemical properties in storage and physiological environment, as well as low cost and widely available of raw materials, PEGylated cationic niosomes are promising drug delivery systems for improved OND potency *in vivo* (Huang et al., 2008).

3.4. In vivo delivery

In the past two decades, various cationic liposomes have been synthesized and used for delivery of nucleic acids in animals and in patients enrolled in phase I and II clinical trials (Dow et al., 2005). The major hurdles that the *in vivo* delivery must overcome include the toxicity and relatively low transfection efficiency of CLs. Strategies to realize the targeting of CLs through topical delivery, conjugation of ligand, control of particles of lipoplexes have been applied to formulate an ideal liposome-based delivery system.

3.4.1. Topical delivery

The topical delivery of liposomes is an attractive strategy for the treatment of diseases occurred in the surface of the body. For instance, they can improve the bioavailability of therapeutic genes delivered to the skin, hair follicle, corneal epithelium and airway epithelium (Domashenko et al., 2000; Toropainen et al., 2007; Meuli et al., 2001; Raghavachari and Fahl, 2002). They are also regarded as an alternative administration route of those therapeutic genes which need to realize the efficacy in the whole body besides the systemic delivery, such as the delivery of gene vaccine through the skin (Cui and Mumper, 2002). Usually, DNA transferred by liposomes was firstly studied *in vitro* using cell culture methods with dividing cells, however, such *in vitro* experiments may be misleading and cause us to overestimate the gene transfer efficiency *in vivo* (Toropainen et al., 2007). Thus, selection of appropriate cell models is particularly important for the precise prediction of the virtue *in vivo* liposome delivery efficiency. In the investigations of liposomes for topical delivery, which differentiated state of the cells is most sensitive to the non-viral gene complexes need to be fully understood in order to select the optimal delivery time of the lipoplexes.

Topical liposomes were used to delivery DNA to mouse skin and to human skin xenografts which resulted in efficient *in vivo* transfection of hair follicle cells (Domashenko et al., 2000). It was demonstrated that the transfection depended on liposome composition, and occurred only at the onset of a new growing stage of the hair cycle. Manipulating the hair follicle cycle with depilation and retinoic acid treatment resulted in nearly 50% transfection efficiency and transgenes administered in this fashion are selectively expressed in hair progenitor cells (Domashenko et al., 2000).

When PEI/DNA polyplexes and DOTAP/DOPE/DNA lipoplexes were used to transfect the dividing, partly differentiated and differentiated human corneal epithelial (HCE) cell line, there were variance of the transgene levels for different complexes and state of cells (Toropainen et al., 2007). PEI was effective in transfecting both the dividing and partly differentiated cells, but ineffective in differentiated cells while DOTAP/DOPE showed high activity in differentiated cell line. When DOTAP/DOPE was chosen for *in vivo* study, a high gene expression level was observed for three days after *in vivo* transfection in the tear fluid and aqueous humor.

3.4.2. Systemic delivery

Due to the size and high superficial charge of the lipid complexes, intravenous delivery of lipoplexes always leads to a rapid deposition in the capillary beds of the lung followed by release into the plasma and ultimate clearance into the spleen and liver (Dass and Choong, 2006). This natural tendency for lung and liver uptake via intravenous administration may be exploited for eradication of pulmonary and hepatic metastases. Such an approach has been taken by several groups with varying degrees of success for pulmonary (Li et al., 2008b, 2005) and hepatic metastases (Zimmermann et al., 2006; Jing et al., 2008; Sato et al., 2007). The surfaces of liposomes always need to be modified by conjugating ligand or antibodies for targeting the specific cell types in the liver (Ko et al., 2009).

Table 1
Intravenous delivery of lipoplexes in animal models.

Target organ	Target gene	Formulation	Model	Characteristic	Refs.
Brain					
Brain	pDNA encoding GDNF	OX26 Mab-PEG-liposomes	Parkinson's disease, rats	Highly efficient	Zhang and Pardridge, 2009; Xia et al., 2008
Lung					
Lung-metastasis	MDM2, c-myc, VEGF siRNA	Anisamide-PEG-liposomes	Lung metastasis-bearing mice	Prolonged animal survival time, low toxicity	Li et al., 2008b
Tumor-bearing lung	pDNA encoding luciferase	Lipoplex (TFL-3)	C57BL/6 mouse bearing lung metastases	Relatively higher gene expression in the lungs	Li et al., 2005
Liver					
Liver ApoB	APOB-specific siRNAs	Liposomes (PEG-C-DMA, DLinDMA, DSPC, cholesterol)	Dyslipidaemias, cynomolgus monkeys	Immediate and lasting efficacy of siRNA treatment	Zimmermann et al., 2006
Liver Kupffer cells	SSL3 against TNF-αsiRNA	Liposomes (PE, CHEMS, cholesterol)	Rat injected with LPS to induce TNF-αsecretion	TNF-α secretion efficiently inhibited	Jing et al., 2008
Liver parenchymal cell	Ubc13–siRNA	Galactosylated cationic liposomes(Gal-C4-Chol and DOPE)	Mouse	Effective hepatocyte-selective gene silencing	Sato et al., 2007
Tumors					
Xenograft tumors	pDNA encoding C/EBPβ	Liposomes (DOTAP, cholesterol)	Nude mice bearing CW-2 human colon tumors	Increased apoptosis in tumors, no apparent toxicity in animals	Sun et al., 2005
Xenograft tumors	pDNA encoding angiostatin K1-K3, endostatin, saxatilin	Anti-TAG-72Fab'-PEG immunoliposomes	Nude mice bearing LS174T colon tumors	Efficient, tumor targeting	Kim et al., 2008
Spontaneous tumors	Canine endostatin DNA, luciferase(control)	Liposomes(cholesterol, DOTIM)	Dogs with cutaneous soft tissue sarcomas	Elicit nonspecific antitumor activity and inhibit tumor angiogenesis	Kamstock et al., 2006
Tumors	Anti-luciferase siRNA	DOTAP/cholesterol liposome, protamine, HA	C57BL/6 mice bearing metastatic B16F10 tumor	Little immunotoxicity silenced 80% luciferase activity in tumors	Chono et al., 2008
Tumors	Luciferase	PEG-Peptide-DOPE conjugate (PPD) MENDs	Nude mice bearing HT1080 tumors	Enzymatic cleavage of PPD in tumors, more specific towards tumors than PEG-MEND	Hatakeyama et al., 2006

Apart from liver and lung targeting, either passive or active targeting, liposomes that have a specificity for the brain were also formulated and achieved good therapeutic efficacy through systemic delivery (Zhang and Pardridge, 2009; Zhang et al., 2009). Plasmid DNA encoding glial-derived neurotrophic factor (GDNF) was encapsulated into Trojan horse liposomes (THLs) with a monoclonal antibody (MAb) to the rat transferrin receptor (TfR). Rats with experimental Parkinson's disease (PD) are treated with three weekly intravenous injections of THLs and near complete abrogation of the neurotoxin effects are achieved at 6 weeks following toxin administration.

It is difficult to realize the heart targeting by simply systemic delivery and to our knowledge there is no successful report by far. However, such a problem may be solved with the introduction of ultrasound related technique. Gas-containing liposome (echogenic liposome) is one of the types of microbubbles which can be loaded with plasmid DNA and esoscillate when sonified by ultrasound. At high ultrasound energies exerted in the heart area, these gas filled microbubble was oscillated and destroyed, releasing the drugs to the environment (Postema et al., 2004a,b). Bubble liposomes have also been used to delivery pDNA or siRNA to tibialis muscles, solid tumor tissues (Suzuki et al., 2008; Negishi et al., 2008). However, there are still some limitations of microbubble liposomes as reviewed by Huang (2008).

A lot of interests have been focused on tumor specificity via intravenous delivery of lipoplexes. Since many *in vivo* experiments were only carried out in rodent tumor models, a study in a large animal spontaneous tumor model was needed. A total of 13 dogs with cutaneous soft tissue sarcomas were enrolled and randomized to receive a series of infusions of lipoplexes containing pDNA encoding either canine endostatin or an irrelevant gene (luciferase). Intravenous infusion of lipoplexes did not result in detectable gene expression in tumor tissues. However, growth of tumors was inhibited in 10 of 13 dogs in treatment period and a significant decrease in tumor MVD was noted in half of 12 dogs. It was hypothesized that the substantial antitumor and antiangiogenic activity of lipoplexes are mainly caused by immune stimulatory properties of lipoplexes (Kamstock et al., 2006). Cases of intravenous delivery of lipoplexes in animal models have been listed in Table 1.

4. Transfection system for non-viral carriers

There is no doubt that the gene carriers play a vital role in the gene delivery, however, some of the parameters other than the gene carrier of the transfection system can also influence the gene expression level. Regardless of the great improvement of the transfection efficiency for non-viral vectors, in most cases, non-viral systems still can not reach the high transfection efficiency as viral vectors yield, nor do they allow long-term transfection. On the other hand, to optimize the transfection efficiency, several other transfection methods, such as using reverse transfection approach and/or 3D systems, were developed. These newly developed systems could multiply the effectiveness of the existed gene carrier and is much easier to use than the synthesis of numerous new materials, which is both time and labor consuming. By changing the transfection system, such as the modifications of the order of adding gene complexes and cells, cell culture environment can be greatly improved, not only in the increased transfection efficiency of the gene carrier, but also in a longer gene expression time. For instance, Holladay et al. (2009) reported that rat mesenchymal stem cells (MSCs) transfected with dendrimer–DNA complexes which were embedded in a cross-linked collagen scaffold showed high transgene expression levels for up to 2 weeks, whereas there is a remarkable decline of transgene expression after it reaching a peak at the third day for the direct delivery. Therefore, the construction of transfection system deserves as much attention as the development of new gene carrier.

Relative to the standard *in vitro* culture system using polystyrene as a gene transfection carrier, the *in vivo* cellular microenvironment can result in a different cell morphology or cell proliferation pattern. The matrix mechanics or fluid transport can influence gene transfer in many aspects. Signaling pathway activity, and ultimately the cellular response, can differ depending on whether the cells are present in a 3D or 2D environment (De Laporte and Shea, 2007). Compared with cultured on 2D substrates, cells cultured in 3D matrixes and scaffolds exhibited a higher rate of cell adhesion, with a similar morphology as shown *in vivo*, and adhere to the matrix through different sets of intergrins (Cukierman et al., 2001). One mechanism by which porous 3D scaffolds increases transfection efficiency may relate to the presence of a large surface area from which to deliver DNA to cells (Jang and Shea, 2003). The 3D scaffold can serve as a reservoir for the complexes without aggregation, which may result in not only higher transfection efficiency, but also a longer expression time of the transgene (Storrie and Mooney, 2006).

When designing a 3D transfection system, several factors need to be considered, including the selection of biomaterials to serve as the scaffold, how to impregnate DNA complex into the scaffold, etc. The scaffold could be either solid polymeric biomaterials or hydrogel made from natural materials. Collagen-based and poly lactic acid are among the most commonly used biomaterials in 3D gene delivery system. Collagen sponges have good biocompatibility but have the drawback of poor mechanical strength for cell proliferation and differentiation. So collagen sponges were reinforced by incorporation of poly(glycolic acid) (PGA) fibers. The *in vitro* culture experiment revealed that the number of MSCs attached increased significantly with the incorporation of PGA fiber as compared with that of the original collagen sponge. A complex of the cationized dextran and plasmid DNA of BMP-2 was impregnated into the scaffolds. When MSCs were cultured in the PGA-reinforced sponge, the level of BMP-2 expression was significantly enhanced in polyplexes impregnated scaffold than bolus delivery in 2D culture method. The alkaline phosphatase activity and osteocalcin content of transfected MSCs cultured in the PGA-reinforced sponge were significantly higher as compared with 2D culture method (Hosseinkhani et al., 2006).

Gene-activated matrix is a term used to describe a cellular scaffold that has been impregnated with genetic transfer elements (Bonadio, 2000). Generally, mechanism by which the DNA is incorporated is catalogued as surface immobilization and scaffold encapsulation (De Laporte and Shea, 2007). The scaffold production procedure involves high temperatures, organic solvents, and the generation of free radicals or shear stresses which may damage the bioactive molecules (Jang and Shea, 2003). The immobilization of vectors to biomaterials, also termed substrate-mediated delivery or reverse transfection, provides a method for gene delivery from scaffolds formed by processes that would normally inactivate the vector if they were encapsulated during fabrication (Bajaj et al., 2001). The gene complexes could be immobilized to biomaterials through non-specific mechanisms, including hydrophobic, electrostatic, and van der Waals interactions (De Laporte and Shea, 2007), as well as specific interactions such as in the form of antigen–antibody or biotin–avidin (Tsai et al., 2007; Uchimura et al., 2007).

Some recent progress in reverse transfection based on 3D structure is displayed in Table 2. In our previous study, a new non-viral vector spermine–pullulan was immobilized on a non-woven fabric of polyethylene terephthalate (PET) with reverse transfection method (Okazaki et al., 2007). The scaffold was treated to be negatively charged to facilitate the adsorption of cationic pDNA–spermine–pullulan complex. MSCs were effectively trans-

Table 2
Reverse transfection systems based on 3D structure.

Scaffold	Vector/gene	Location/species(application)	Result	Ref.
PLGA	Transfast/pDNA encoding luciferase and β-galactosidase	Injured rat spinal cord	2-fold greater transgene than naked plasmid, sustained expression	De Laporte et al., 2009
PLGA	PEI/pDNA encoding luciferase	*In vitro* on DC	High levels of expression for 10 days, *in vitro*	Ali and Mooney, 2008
PLGA	[14]/pDNA encodingβ-galactosidase	HEK293T trafected *in vitro*	Large numbers of transfected cells (>60%) at low surface quantities (<50 ng)	Jang et al., 2006
POC	PEI/pDNA encoding luciferase	Intraperitoneal fat model, HEK293	Polyplex loaded-scaffolds successful *in vitro*, but no better than naked pDNA-containing scaffolds *in vivo*	Zhang et al., 2009
MMP-degradable hydrogels	PEI/pDNA encoding GFP, luciferase	MSC trafected *in vitro*	Different profiles for clustered and homogeneously seeded cells.	Lei and Segura, 2009
Type I/III collagen scaffold	Lipoplexes/pDNA encoding endostatin	Angiogenesis, *in vitro*	Potential of sustained anti-angiogenic protein release	Sun et al., 2009
PGA-fibers reinforced collagen sponges	Dextran-plasmid/pDNA encoding BMP-2	MSC transfected *in vitro*	Significantly higher ALP activity and osteocalcin content of transfected MSC in 3D than in 2D culture method	Hosseinkhani et al., 2006
Collagen scaffold	dPAMAM (SuperFect™, Qiagen), pDNA encoding luciferase	rMSCs, ADSC, PC-12, PC-12 differentiated, HL-60, Jurkat, VIC, Osteosarcoma, HUVEC, HUASMC trafected *in vitro*	Elongated tranfection period and relatively high gene expression over bolus gene delivery on rMSCs, ADSC, inefficient for HL-60, Jurkat	Holladay et al., 2009
Collagen sponge	PEI, DOTAP/cholesterol, PEI-PROCOP, pDNA encoding luciferase	Subcutaneous implantation in rats	Gene expression last for at least 3 days for DOTAP/cholesterol-DNA and PEI-DNA-loaded implants. PEI-DNA-PROCOP collagen yielded the highest gene expression levels for at least 7 days	Garcia et al., 2007
Chitin and alginate	PEI/pDNA encoding bFGF, GFP	HEK293T, human dermal fibroblasts trafected *in vitro*	Significant transgene expression on scaffolds even after 2 weeks of culture	Lim et al., 2006
Hyaluronic acid-collagen hydrogels	PEI/pDNA encoding luciferase	NIH/3T3 trafected *in vitro*	Transgene expression significantly affected by Complex size, Spatially controlled gene transfer achieved by topographically patterning the hydrogel	Segura et al., 2005

fected in this 3D system, and the level of gene expression was significantly higher than MSCs transfected by culturing in the medium containing the complex (conventional method). It is generally accepted that gene incorporation through scaffold encapsulation has the capacity to deliver large quantities of gene complexes and release sustained over a long period (Jang et al., 2005; Bonadio et al., 1999), compared with substrate-mediated delivery which mostly significantly less DNA could be incorporated. The gene transfer levels in the 3D transfection system are dependent on the interactions among the cells, scaffold and gene complexes.

4.1. Cell-scaffold interactions

4.1.1. Cell types

For certain types of cells, the transfection levels are higher in 3D than in 2D environment, such as the case in HEK293T, MSC, ADSC, DC (Holladay et al., 2009; Ali and Mooney, 2008; Jang et al., 2006; Lei and Segura, 2009). It is possible that primary cells are more sensitive to matrix interactions, proliferated quickly in 3D scaffold, and thus the 3D environment act as a more powerful adjuvant for these cells. However, for some cell lines, such as NIH/3T3 which itself is not difficult to be transfected as reported by Tatiana Segura, which is less efficiently transfected in 3D than 2D bolus gene delivery, even though the optimization of particle size, etc. (Segura et al., 2005). There are still some cells that are difficult to be transfected in both 2D and 3D environment, such as monocyte cells HL-60, Jurkat and S180 which are either suspension cells or differentiated cells. So whether the 3D transfection method could increase the gene expression levels of a specific cell is still waiting to be discovered.

4.1.2. Cell distribution

In some conditions such as the tissue regeneration, spatially uniform distribution of cells is required. Cell-seeding method affects the cell distribution in the scaffold. For solid scaffold, cells are commonly seeded through injections of cell suspensions with a high density and agitation can also be used to promote the infiltration of the cells (Takahashi and Tabata, 2003). As for the hydrogel scaffold, cells can be seeded through homogeneous mixture with the hydrogel or in the clustered form (Lei and Segura, 2009; Dadsetan et al., 2009) and which method to use varies from case to case. It was reported that the cumulative transgene expression of MSCs seeded in matrix metalloproteinase (MMP) degradable hydrogels in the Clustered form kept increasing through the 21-day incubation, while the cumulative transgene expression of the homogeneously seeded cells plateaued after 7 days of culture. The MMP activity is highest at the cell surface, so the gene complex is hardly released from the hydrogel until the cell migrates there. It was explained by the author that the clustered cells possess a better migration ability and thus internalize more gene complexes released (Lei and Segura, 2009).

4.1.3. Mechanical property of the scaffold

The mechanical properties of the scaffold play a role in the proliferation of the cells and can indirectly affect the gene transfer, since the cell division have been reported to be able to enhance the gene transfer for non-viral vectors (Escriou et al., 2001). Kong et al. (2005) prepared alginate hydrogels with various mechanical properties and incorporated PEI/DNA complex into the hydrogels to transfect MC3T3-E1 cells. It was demonstrated that the elastic moduli of the hydrogels could mediate cellular proliferation and apoptosis. The transgene expression exponentially increased as the shear modulus exceeded a certain level, indicating the importance of mechanical stiffness of the scaffold. However, the mechanisms underling the effects have not been elucidated.

4.2. Scaffold-complex interactions

The *in vitro* experiment of DNA release from the scaffold can not exactly reflect the release pattern in the real transfection system as cells are not involved in the release experiment, but it leave us some cues of the barriers we need to pay attention to. In some cases, burst release is severe for hydrogels made of synthetic materials (Chun et al., 2005), while for natural materials the release is relatively slow but incomplete (Megeed et al., 2004). These two problems can cause the waste of DNA and the limited gene expression level and course. An alternative strategy is to combine synthetic materials with natural materials. For example, the burst release of the DNA from Pluronic F127 hydrogels was remarkably reduced by the combination of di-acrylated Pluronic F127 with vinyl group-modified hyaluronic acid (HA) (Chun et al., 2005).

4.3. Cell-complex interactions

The physical properties of the gene complex, such as the particle size and surface charge, significantly affect its interactions with the cell surface. There is a tendency to pursue the nanoparticles whose particle size is around several hundred nanometers or less, as this particle size could facilitate the cell endocytosis in some cases and thus increasing the numbers of particles in cytosic plasma. However, relatively bigger particles of micrometers are not necessarily less effective in 3D substrate-mediated gene delivery than small particles. In the research carried by Lonnie sea et al. (Segura et al., 2005), gene expression levels were higher for the immobilization of bigger PEI/DNA complexes, but the percentage of cells transfected was higher than the smaller complexes. The influence of particle size may be a function of change of the uptake and subsequent intracellular pathways, the two aspects that could alter the toxicity and fate of the nanoparticles. The inclusion of the biomaterials of the scaffold, the interactions of each part may further increase the complication of this problem.

5. Conclusions and perspectives

In summary, various strategies have been introduced to refine the non-viral vectors, including the development of composite carrier materials or biodegradable polymers with reduced toxicity, incorporation of cell targeting and additional transport domains for effective and specific delivery, combination of cationic polymers with lipids to facilitate endocytosis and so on. With respect to the transfection approaches, there is a tendency to mimic the microenvironment of tissues by transferring the conventional 2D substrate to 3D matrix for a better investigation of non-viral vectors. Continued studies of either viral or non-viral carriers' cellular processes and development of the structure-function relationships will provide a better guideline for the design of non-viral vectors in the molecular scale. In addition, the combination of scaffold with non-viral gene transfection technology will also accelerate the clinical application of non-viral gene delivery systems.

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