Cationic Polymer Optimization for Efficient Gene Delivery

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Abstract: The polyplexes which are formed between cationic polymers and DNA through electrostatic interactions and thus known as polycation/DNA complexes, are by far the most widely used non-viral gene delivery vectors. Many factors such as molecular weight, surface charge, charge density, hydrophilicity and the structure of cationic polymers affect gene transfection efficiency of cationic polymers. Therefore, optimization of cationic polymers is necessary to improve the gene transfection efficiency. Currently several important cationic polymers were used as cationic vectors for gene delivery which included PEI, PLL, Chitosan and PAMAM. Their most advantages and the rational design are introduced in this article. However, these systems are much less efficient in gene transfer experiments compared with viral systems. Some strategies such as PEGylation, combination and multifunctional modification were developed in the cationic polymeric vectors for gene delivery. Hereby, this article will review various kinds of copolymers with higher stability but biodegradable, bioresponsive and easy refined molecular weight which could be easily modification. Especially, the multifunctional modified polyplexes and polymersomes will be further discussion due to their ability to conjugate biologically active ligands, which can be used as potential nanostructured biomaterials for future in vivo gene delivery.

Key Words: Cationic polymer, polyplexes, gene delivery, optimization.

1. INTRODUCTION

Recently, the studies on gene delivery by using cationic polymer have been experiencing a growing interest owing to the appearance of clinical protocols for gene therapy [1,2]. Cationic polymers are high molecular polymers with positive charges on the surface, which interact with negatively charged phosphate in DNA backbone through electrostatic interactions. These electrostatic interactions were formed between the negative phosphates along the DNA backbone and positive charges displayed on the polymer material, leading to polyplexes [3]. The formation of polyplexes is spontaneous and entropically driven.

In such systems, much greater flexibility can be achieved simply by varying the composition of the mixture, the cationic polymers' molecular mass, architecture (linear, randomly branched, dendrimer, block and graft copolymer) and through modification of their backbone by the introduction of side chains or target-specific ligand [4]. Polyplexes are also relatively inert and biocompatible, while their biological behavior can be regulated by controlling the size and surface properties and the release of genetic materials can also be controlled by changing the degradation rate of the matrix polymer [5]. Besides, the polyplexes can neutralize the negative charge of DNA plasmid, condense the large space capacity of DNA, enhance the system penetrating, and can embed the DNA to protect DNA from degrading by nucleinase. The excess positive charge on the polyplexes surface could attach to the target cell surface, be internalized, escape from endosomes, find a way to the nucleus, finally, be available for transcription.

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However, there will be hurdles to make polyplexes suffer from low gene transfer efficiency in each step [6]. It must be considered the extracellular barriers such as plasma membrane, endocytosis and intracellular barriers to traffick as internalization, endosomal escape and nucleus entry when rational design cationic polymers. The other obstacles in the use of polyplexes are their aggregation, instability, toxicity and their propensity to be captured by the mononuclear phagocyte system (MPS) [7]. The high charge density on the surface of ploxplexes contributes to their cytotoxicity [8], which include nonspecific membrane destabilizing effects and intracellular polyplexes mediated toxicity [9]. Also the unspecific binding to non-target tissues or inefficient uptake into target cells, limited releasing from endocytotic vesicles, and ineffective import into the nucleus of target cells limit the development of ploxplexes [10]. So PEGylation strategy to decrease the cytotoxicity and multifunctional strategies have been widely investigated.

As the improved material industry and synthetic chemistry for optimizing polymers with uniform size, topology and transport domains, a better understanding of the mechanism of barriers, endocytosis, gene transcription, the detailed structure-activity relationship of sequence-defined polymers and multimodal imaging methods in experimental animals and human subjects *in vivo*, cationic polymers or multifunctional polyplexs could be developed as ideal vectors because of the controlling of the various properties of polymers and assigning various kinds function of polymers would become a reality. Furthermore, dynamic, bio-responsive polymers will cope better with the multiple sequential delivery steps [11].

This review provides an overview of the most frequently used cationic polymers interact with DNA leading to polyplexes in non-viral gene delivery, describing the specific advantages and disadvantages of several important classes of

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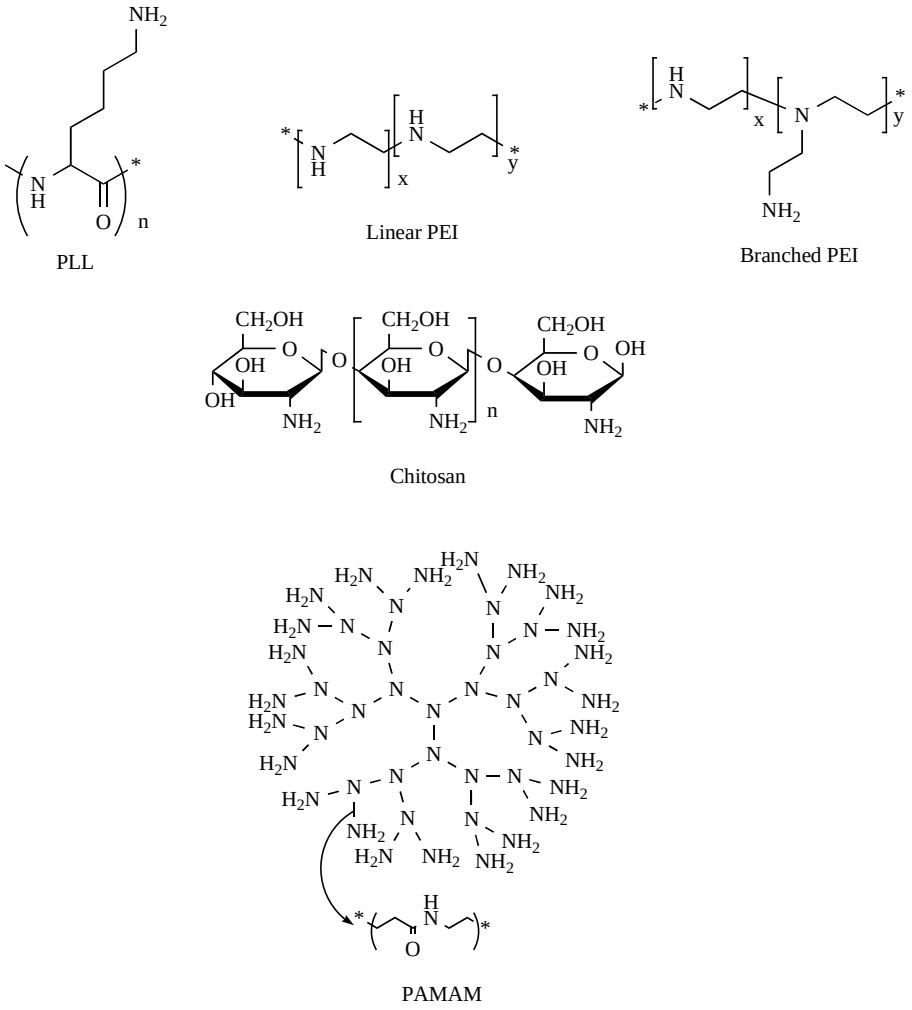

Fig. (1). Structure of PLL, branched PEI, linear PEI, chitosan and PAMAM.

cationic polymers, and the strategies for PEGylation to reduce toxicity and to produce a shielding effect that counteract effective DNA complexation; rational design various kinds of cationic polymers and multifunctional polyplexes with defined size, topology and transport domains obtain efficiency gene delivery vectors *in vitro* and *in vivo*.

2. THE COMMONLY USED CATIONIC POLYMER

Cationic polymers generally interact with DNA to form small complexes with a net positive surface charge. The commonly used cationic polymers include nature polymers such as chitosan or synthetic polymers such as, poly (L-lysin) (PLL), polyethylenimine (PEI), and dendrimers.

2.1 PEI

Polyethylenimine (PEI), an organic branched, linear or dendrimer polyamine polymer, has been successfully used for DNA complexation and transfection *in vitro* and *in vivo* into several cell lines and tissues in the past [12]. Acid-catalyzed polymerization of aziridine leads to branched PEI, where as ring opening polymerization of 2-ethyl-2-oxazoline results in the N-substituted polymer. This process can be transformed *via* hydrolysis into linear PEI [13,14], their molecular weights usually range from low-molecular weight (<1000 Da) to high-molecular weight (>1000 KDa).

PEI which has been used as a gene delivery vector by Boussif *et al*. since 1995 [15], is the most used cationic polymer with high cationic potential. But at present, the synthesized PEI derivatives have a few of advantages over PEI. In the studies by Forrest *et al.*, ester cross-linked PEI derivatives were prepared by reacting PEI 800 Da with small diacrylates, this synthesized polymers mediated gene expression more efficiently than PEI 25KDa. Furthermore, the ester cross-linked PEI derivatives did not have cell toxicity and the ester bonds were susceptible to hydrolysis [16].

Among the cationic polymers, PEI offers the advantage of efficient endosomal release due to its high pH-buffering capacity. PEI, which contains a large number of secondary and tertiary amines and exhibits pKa values between physiological and lysosomal pH, has been termed the 'proton-sponge' polymer. The 'proton-sponge' hypothesis is used to explain endosomal disruption by cationic polymers with ionizable amine groups. The 'proton sponge' nature of PEI is thought to lead to buffering inside endosomes [17]. Endosomes are acidified by the action of an ATPase that actively transports protons from the cytosol into the vesicle. The accumulation of protons in the vesicle could be balanced by

an influx of counter ions. At last, the increased ion concentration causes osmotic swelling and physical rupture of the endosome membrane, resulting in polyplexes release into the cytosol [18]. PEI makes an excellent proton sponge because of its very high density of amines. The proton sponge hypothesis, also has been explained the relatively high transfection efficiencies of other proton-sponge-type polymers, such as histidylated poly-L-lysine [19], poly (amido amine) (PAMAM) dendrimers [20], lipopolyamines [21], and various imidazole-containing polymers [22].

The high transfection efficiency of PEI might be explained by its buffering capacity and large part to efficient escape from the endocytic pathway through the proton-sponge mechanism [23,24]. However, degradability, cytotoxicity, aggregation, and short circulation time in the bloodstream *in vivo* still remain. So it is still necessary to design novel PEI derivates or modified PEI.

2.2 PLL

Poly-l-lysine (PLL) is one of the first polymers which used for DNA condensation and continues to be applied in gene therapy widely [25]. The primary ε-amine groups of lysine in PLL, could electrostatically interact with negatively charged phosphate groups of DNA to form polyplexes. The first studies on PLL mediated gene delivery showed that PLL conjugated the asialoorosomucoid glycoprotein could target the asialoglycoprotein receptor on mouse hepatocytes *in vitro* and *in vivo* [26]. Later, Wagner and colleagues reported that conjugation of the iron transport protein was able to transferrin to PLL [27]. Although the early studies on polylysine seem promising, PLL-based polyplexes had lots of limitation in clinical applications because of their relatively low efficiency. Now, the modified PLL were caught much concern for PLL with the reactive primary amino groups of the side chains of the lysine residues, which could be utilized to introduce various functional groups such as hydrophobic groups [28], thiol groups [29], and histidyl moieties [30].

2.3 Chitosan

Chitosan, obtained by deacetylation of chitin, a biodegradable polysaccharide composed of two subunits, d-glucosamine and N-acetyl-d-glucosamine which are linked by a (1→4) glycosidic linkage, has an average molecular weight ranging between 4 and 20 KDa [31]. Chitosan could also establish electrostatic interactions with the negatively charged DNA to form polyplexes.

The many advantages of chitosan, include safety, biodegradability, ease of modification, ease of DNA complex formation, widespread availability, low cost, with high cationic potential. So chitosan and chitosan derivatives have been widely proposed as alternative, low toxicity and biocompatible cationic polymers that make them an ideal candidate for gene delivery strategies [32]. But the low specificity and low transfection efficiency of chitosan must be overcome for its use in clinical trials.

2.4 PAMAM

The most currently used dendrimers are polyamines, polyamides or polyesters, but the most commonly encountered is PAMAM because of its high transfection efficiency. PAMAM dendrimers are spherical, ordered, branched polymers with positively charged amino groups on their surface and also tertiary amines in the branches [33].

As early as 1984, PAMAM dendrimers were the first complete dendrimer family (G=0-7) to be synthesized and characterized, followed by commercialization in 1990 [34]. Haensler and Szoka originally reported the use of PAMAM dendrimers for gene delivery [35]. Then PAMAM dendrimers modificated with NH_2 group, had been most widely studied for medical applications in gene delivery [36]. The dendrimers which had two major strategies through michael addition of methyl acrylate and the amine core had evolved for dendrimer synthesis. The first was divergent method in which growth of a dendron originated from a core site which involved assembling monomeric modules in a radial, branch-upon-branch motif according to certain dendritic rules and principles, and the second method followed a convergent growth process that proceeded from what would become the dendrimer surface inward to a reactive focal point, leading to the formation of a single reactive dendrimer [37,38].

A vast array of dendrimer chemistries and hybrid dendritic architectures were currently being studied, even more complexs array of dendritic architectures have emerged. Structures reported include dendritic hybrids, dendronised polymers, dendrigrafts, cyclodextrin-dendrimer hybrids, core-shell architectures, cascade-release dendrimers and self assembling dendrisomes (Fig. (**2**)) [36].

The advantages of PAMAM dendrimer are obvious, including their nanoscale spherical architecture (at higher generation), narrow polydispersity and the multifunctional surface offering the possibility to tailor-make their surface chemistry. The relatively empty intramolecular cavity could be amenable to a host-molecule entrapment providing opportunities for subsequent controlled drug or gene release. Meijer *et al.* proposed the notion of a "dendritic box" [39].

PAMAM dendrimers as nanoscale containers contain multivalent surface, the interior shell and the core to which the dendrons are attached. The core-shell structures represent well-defined nano-environments, which are protected from the outside by the dendrimer surface in the case of higher generation dendrimers [37]. The core-shell structures might also be described as micelle mimetics behavior of dendrimer.

Based on the advantages and their structure disadvantages of these cationic polymers, it is necessary for chemical modification and rational design various kinds of cationic polymers to improve transfection efficiency and reduce cytotoxicity.

3. INFLUENCE FACTORS OF CATIONIC POLYMER ON GENE TRANSFECTION

Many factors affect gene transfection efficiency such as cationic polymeric category, molecular weight, surface charge and charge density, backbone structure of cationic polymers, these factors have been shown to affect DNA condensation into polyplexes and induce aggregation, toxicity and buffering capabilities of the polyplexes. The physicochemical properties of cationic polymers are critical

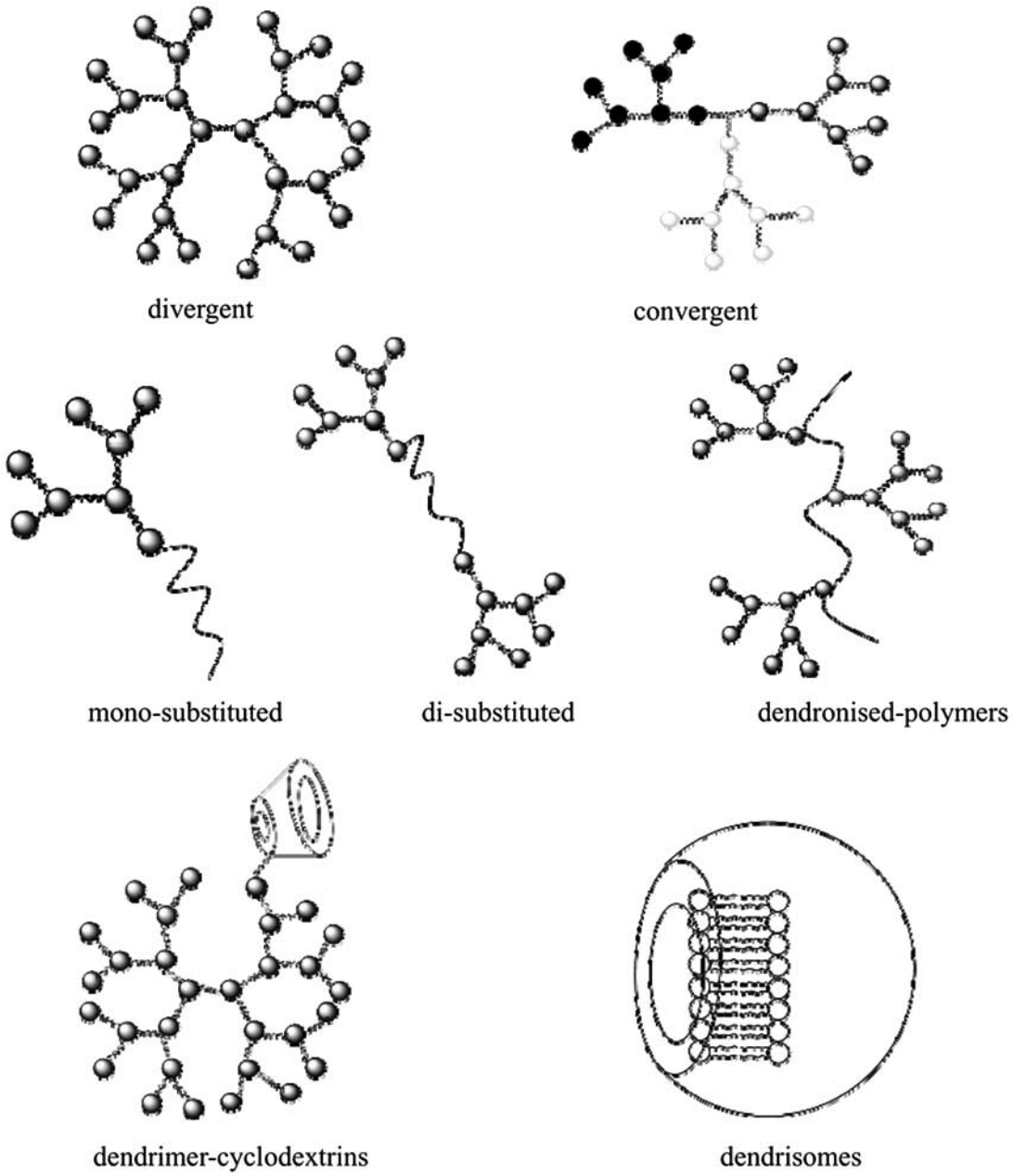

Fig. (2). Diagram showing schematically dendritic architectures under development for biomedical.

factors that govern complex distribution, bioavailability and the gene expression profile *in vivo*. According to the influence factors of gene transfection, chemical modification with cationic polymers backbone has been studied and researched constantly.

3. 1 Molecular Weight

The transfection efficiency and cytotoxicity of certain cationic polymers are dependent on molecular weight (MW) greatly. Usually, high molecular weight (HMW) cationic polymers had efficient gene transfection but also induced cytotoxicity, while low molecular weight (LMW) cationic polymers could reduce cytotoxicity but had poor gene transfection. The transfection efficiency of polyplexes formed with both PEI and PLL increased with increasing molecular weight, but HMW also resulted in increased toxicity, limiting effectiveness both *in vitro* and *in vivo* [40].

PLL with a molecular weight of less than 3000 could not form stable complexes with DNA, it was showed that the number of primary amine in the PLL backbone was important for the polyplexes formation, and DNA condensation ability and transfection efficiency increased with increasing of molecular weight of PLL [41].

PEI of molecular weight around 22-25 KDa mediated gene transfer very effectively probably due to efficient endosomal escape. However, it has considerable toxicity because it couldn't degrade into small degradation products. LMW PEI was less toxic but showed almost no transfection [42]. Simultaneously, a novel low-molecular weight polyethylenimine 'PEI F25-LMW' was derived by gel permea-

tion chromatography with a low molecular weight (~4-10KDa) and high delivery efficacies for high DNA and siRNA. It shows low toxicity in various cell lines and different conditions [43]. Furthermore, PEI F25-LMW-based complexes could be kept frozen for several months without loss of transfection efficacy and thus might represent a long-term storage, ready-to-use formulation of DNA or siRNA based gene therapy products [44]. The higher transfection efficiency of PEI F25-LMW compared to other LMW PEI might be due to PEI F25-LMW had a narrower fractionation, and in the process of preparation the vast majority of PEI molecules of higher molecular weight which displayed no or almost no transfection efficacy was removed. Besides, these complexes were considerably small size, which would induce more efficient uptake and lower cytotoxicity *in vitro*, while the commercial PEI formed large aggregates on the cell surface and impaired membrane functions, finally leaded to cell necrosis [43].

3.2 Charge Density

High charge density of the cationic polymers system increases the transfection efficiency, but it simultaneously contributes to increased cytotoxicity. In fact, the efficacy of transfection is a sweet compromise between the transfection efficiency and the cytotoxicity. Charge is a key parameter assumed to play an essential role and also is known to have an influence on the cytotoxicity and the barrier integrity [45]. For example, high charge density of PEI contributed to the formation of highly condensed particles by interacting with DNA. However, the property might cause significant cytotoxicity. But the cell cytotoxicity and transfection efficiency of PEI, were dependent not only on charge density but also on molecular weight of the polymer [46].

3.2.1 Acetylizad Modified Polymers Reduce Positive Charge

Increased transfection efficiency through reduction of positive charge density has been widely reported. Partial acetylation of PEI amino groups is shown to enhance gene transfer activity and further N-acylation of PEI could diminish its toxicity, this may be due to acetylation neutralizes charges and enhances dissociation of polymers inside of the cell [47,48].

The polyplexes formed with acetylated PEI were more easily dissociated into free polymer and DNA compared with unmodified PEI, which suggested that low positive charge of acetylated complexes reduce the electrostatic interaction between polymer and DNA. Cellular proteins or negatively charged cellular or endosomal membranes could pull the acetylated polymers away from the negatively charged DNA more easily, leaving a partially uncomplexed DNA free in the cytosol. The reduced surface charges meaned the polymer was also less likely to be toxic [47]. Gene-transfer activity increased upon acetylation, and the polymer with acetylation on 43% of the primary amines was as much as 26-fold more efficient than unmodified PEI. Besides, acetylation had other effects on such as the reductions of the average pKa and buffering capacities of the polymers, these changes in polymer protonation might influence the endocytic trafficking of the polyplexes and their escape into the cytosol. Ultimately, acetylation might impact the lipophilicity of the PEI, which enhanced gene transfection efficiency [48].

Thomas and Klibanov had systematically introduced chemical modifications of the PEI nitrogen atoms, acetylation or dodecylation systematically, which formed PEI derivatives markedly enhanced gene transfection. The dodecylation of primary amino groups of 2 KDa PEI that presenced of long lipophilic substituents on PEI could increase the interaction of PEI/DNA complexes with cell membrane, yielded a nontoxic polymer whose transfection efficiency in cell culture was 400-fold enhanced [49].

Waite had also reported acetylation of PAMAM dendrimers for cellular efficient delivery of siRNA. Primary amine acetylation of PAMAM dendrimers reduced cytotoxicity to U87 cells, and promoted the release of siRNA from dendrimer/siRNA complexes [50]. Patil and colleagues reported novel, surface neutral and internally cationic PAMAM generation four dendrimers for the efficient intracellular gene delivery, the surface primary amine groups of PAMAM-NH_2 dendrimer were modified by acetylation followed by internal quaternization. The developed dendrimers possessed advantages of neutral outer surfaces of the dendrimer for low cytotoxicity and existence of cationic charges inside the dendrimer not on the outer surface that resulted in highly organized compact nanoparticles, which could potentially protect nucleic acids from degradation [51].

3.2.2 Quaternary Amine Groups with Chitosan Maintain Permanent Charge

It has been reported that the transfection efficiency of quaternary chitosan was affected by the degree of quaternization and quaternary chitosan. Quaternary ammonium derivatives of chitosan showed higher solubility over a broader pH range and more permanent positive charge on the polysaccharide backbone than those unmodified chitosan [52].

Kean *et al.* prepared N,N,N-trimethylated chitosan polymer (TMC) and N,N,N-trimethylated chitosan oligomer (TMO) (Fig.(**3**).) by quaternization on chitosan backbone. The derivatives complex pGL3 luciferase plasmid DNA was able to transfect MCF-7 cells with greater efficiency than PEI 25 KDa. The study showed that increasing amount of trimethylation might confer more permanent charges to the polymer and also extend the range of pH over which the polymer was soluble. This had the probable effect of increasing the interaction with pDNA. The transfection efficiency with increasing degree of trimethylation of TMO indicated that the highest efficiencies were around 44% of trimethylation, possibly because the optimum complexes were made around its pKa of 6.2-6.5. Meanwhile the percentage of trimethylation of TMO showed little or no toxicity. The increasing the degree of trimethylation could increase the toxicity until polymers trimethylated to 93%, which did not follow this trend and display less cytotoxic. This might be explained that 93% trimethylated need to extend time of derivatisation reaction, thus might cause the polymer to break up, forming smaller fragments which led to lower toxicity. However, higher toxicity was investigated in polymeric chitosan derivatives over oligomeric chitosan derivatives at similar degrees of trimethylation, it was

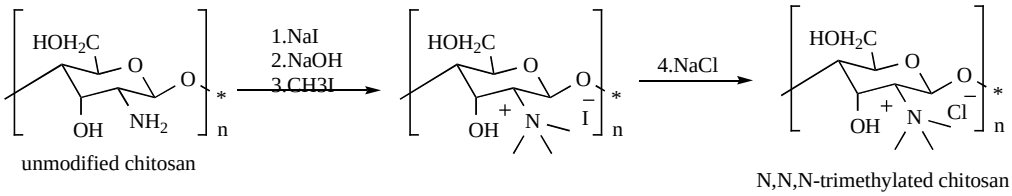

Fig. (3). Reaction scheme of chitosan trimethylation [53].

possibly explained that the increased ability of the cell to expel the dissociated (from pDNA) oligomer was easier than the polymer and fewer contacted points of each individual oligomeric chain could interact with the cell components [53]. Chitosan N-betainates (Fig. (**4**)) which had a quaternary ammonium group, with betaine substitution increasing, the transfection efficiency increased in COS-7 cells, and cellular uptake also increased because the increasing of the substitution degree of betaine could increase the positive charge on the complex, besides the quaternary chitosan was capable of opening tight junctions of cells that could increase paracellular primeability [52].

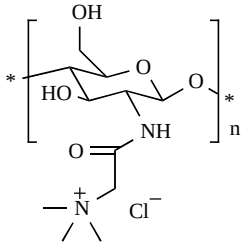

Fig. (4). The structure of Chitosan N-betainates.

3.3 Biodegradablity

The cytotoxicity of biodegradable polymers is lower than non-biodegradable polymers. Natural degradable polymer as chitosan, which can be degraded by chitinase and lysozyme, is much less toxic than non-degradable polymer PEI [54]. Chemical modified non-biodegradable polymer is profitable to hold the same or enhance gene transfection efficiency while reduces cytotoxicity at the same time. Many strategies such as synthesis of HMW polycations from LMW oligocations *via* biodegradable linkages, could reduce the toxicity of the polyplexes while retain their stability in physiological conditions.

Several investigators synthesized cationic polymers consisting of LMW PEI and degradable cross-links. Disulfides, esters, acetals and orthoesters are the most widely used among many possible biodegradable bonds. The disulfide bonds are stable in the oxidative extracellular condition and can be degraded rapidly in the reductive intracellular condition. Kissel *et al.* prepared reversibly stabilized 25 kDa bPEI/DNA polyplexes *via* crosslinking with a LMW amine reactive cross-linker dithiobis(succinimidyl propionate) (DSP). The cross-linking after polyplex formation was able to enhance resistance against polyanion exchange and high ionic strength [55]. Qi Peng *et al.* had investigated the influence of disulfide density and molecular weight on disulfide cross-linked polyethylenimine, it was shown that the polymers with moderate thiolation degrees of 2.6, 3.5, and 4.5 forming the most compact polyplexes had highest efficiency, the polymers with very low or very high thiolation degrees were unable to form compact polyplexes and had very poor transfection efficiency [56].

Cationic polymers crosslinked hydrolyzable esters have been established to effectively transfer gene material into cells while produced substantially lower cytotoxicity because of their rapid hydrolysis into nontoxic metabolites. LMW PEI (800 Da) connected *via* ester bonds with 1,3-butanediacrylate or 1,6-hexanediacrylate cross-linkers degraded at physiological conditions with half-lives of 4 h and 30 h, respectively. The cytotoxicity of the polymers/DNA was significantly reduced (cell viability around 85%) compared to those non-degradable 25 KDa PEI (cell viability 50%) in C2C12 cells, while mediated gene expression 2- to 16-fold higher than 25 KDa PEI [57]. Polyglutamic acid is a biodegradable polypeptide, the biodegradable backbone poly (amino acid) derivates (poly(ethylene glycol)-blockpoly(γ-benzyl L-glutamate) PEG-b-PBLG) was designed, then attach the LMW PEIs (MW 423 Da) to the side chains of poly(amino acid)s by the reaction of aminolysis. The study indicated that the modification of PEI of grafting LMW PEI to the main chain of PEG-PLG to form a LMW PEI combined copolymer with suitable HMW could ameliorate the DNA condensation ability of the polymer. The DNA condensation capability was increasing with the increasing amino density upon increasing the PLG length as well as the increased PEI molecular weight of the polymer [58].

4. STRUCTURAL MODIFICATION AND THE STRUCTURE-ACTIVITY RELATIONSHIP

Different cationic polymer structure would show different transfection efficiency. Studies with linear PEI showed even higher transfection efficiency and lower cytotoxicity compared to branched PEI [59,60]. However, the transfection efficiency of branched PEI with similar molecular weight could increase by decreasing the degree of branching [61]. PAMAM dendrimers with high structural flexibility and partially degraded high-generation such as hyper branched architectures appeared to be better suitable for certain gene delivery than intact high-generation symmetrical dendrimers [62], because partially degraded PAMAM dendrimers had more flexible structures than intact dendrimers and therefore to interact more efficiently with DNA [63]. A fragmentation step consisting of hydrolytic cleavage of the amine bonds could enhance transfection efficiency [64]. Different chemical structural modification on polymers would improve gene delivery efficiency by different mechanisms.

4.1 Chemical Structural Modification to Facilitate Bioresponsive Strategies

Bioresponsive polymers could adapt to the different delivery barriers, respond to external physical stimuli, carry inherent own therapeutic activity in addition to the nucleic acid part, will efficiently deliver nucleic acid into cells at various steps of delivering while limiting effects of cytotoxicity.

Cationic polymers with bioresponsive strategies such as chemical bond cleavage might promote reversion of the high molecular weight transfection complexes back to low molecular weight counterparts, and the low molecular weight fragments could be cleared more easily from the body respecting enhanced diffusion and potentially easier filtration through glomerular capillaries. The following endocytosis and subsequent endosome release, the polyplexes formed between cationic polymers with disulfide bonds and DNA in the extracytoplasmic environment, could induce the efficient release of entrapped DNA from the polyplexes after their movement into the reductive cytoplasmic compartment. This would achieve efficient gene expression *in vivo* while limiting effects of toxicity [65,66].

4.1.1 Cross-link Disulfide Bonds

Cationic polymers bearing disulfide bonds have bioresponsive strategies in the intracellular reducing environment. The disulfide bonds is relatively stable under physiological conditions, but can be destroyed by the reducing agent glutathione. The cationic polymers with disulfide bonds are stable during systemic circulation, but dissociate in reducing endosomal and cytosolic environments, thus delivery DNA effectively into the cytoplasm and nucleus [67].

PEI with degradable linkages by disulfide bonds may produce high gene transfection efficiency while reduce cytotoxicity. For example, the degradable crosslinked PEI-derived gene vectors were obtained via crosslink low molecular weight PEI (800 Da) with disulfide-containing crosslinkers, such as dithiobis (succinimidylpropionate) (DSP) and dimethyl 3,3V-dithiobispropionimidate (DTBP), exhibited higher transfection efficiency and lower toxicity (Fig. (**5**)). The introduction of disulfide bonds through DSP and DTBP might promote reversion of the high molecular weight complexes back to low molecular weight counterparts. The intracellular reduction of polymer disulfide bonds could lead to the release of DNA for nuclear uptake and transcription. Besides, using cross-linked polymers would alleviate an accumulation of PEI localized in the nucleus, accordingly, evaded PEI interacted with endogenous DNA that potentially led to undesirable side effects. [65] Kloeckner also synthesized different cationic polymers by oligomerization of low molecular weight oligoamines with three different crosslinkers, DSP, DTBP and hexanediol diacrylate (HD) to generate degradable polycations with

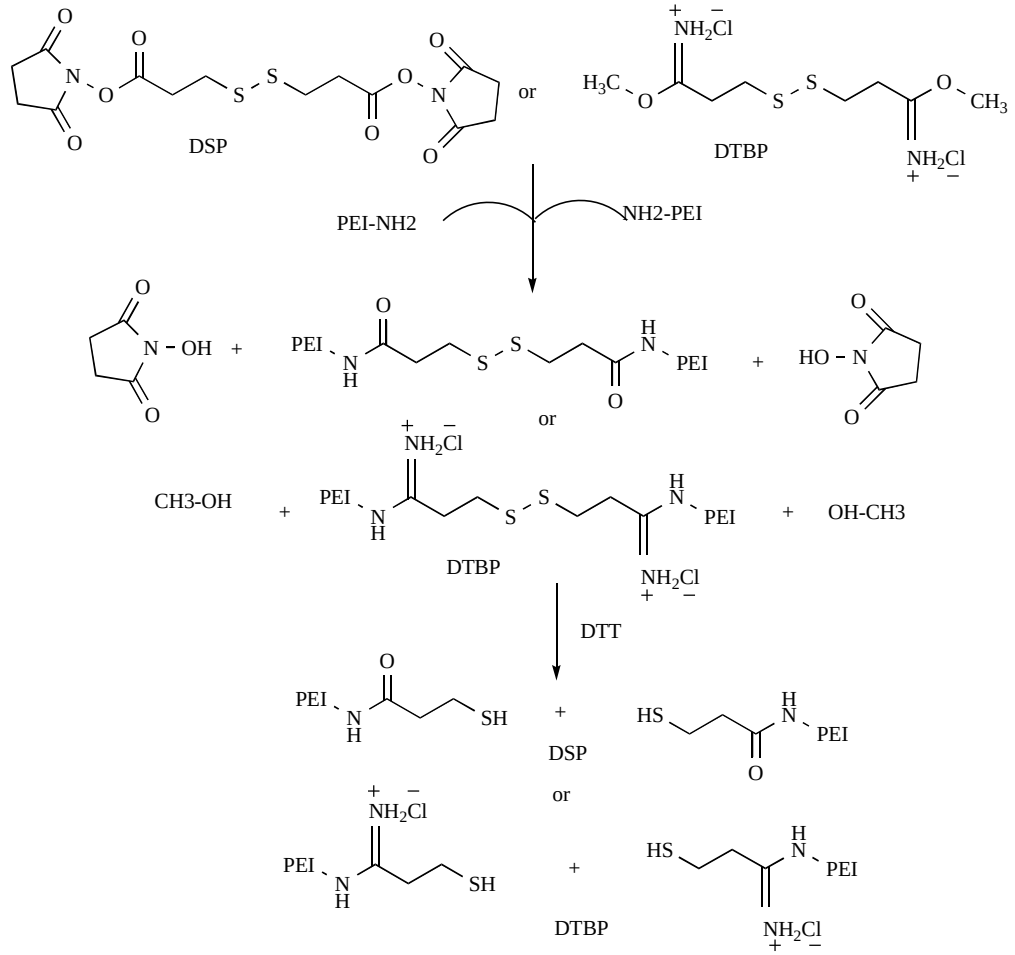

Fig. (5). Reaction scheme for conjugating PEI with cross-linking reagents DSP and DTBP.

sufficient cationic charges for DNA condensation. The results showed that oligoethylenimine (800 Da)-based polymers possessed the highest gene transfer efficiency and exceeded the golden standard linear polyethylenimine (22 KDa) in terms of activity or toxicity profile [68].

PLL is incapable of medicting efficient escape from endocytic vedicles into the cytoplasm, and poor gene expression is seen *in vitro* without endosomolytic agents as chloroquine. PLL-based degradable cationic polymers by introducing bioreducible disulfide bonds have been caused much concern. Kataoka investigated disulfide cross-linked polyion complex micelles of PEG-PLL and DNA, the disulfide bonds were reduced by high intracellular concentrations of glutathione, thus destabilized the complexes and released the DNA [69]. Oupicky *et al.* also demonstrated that crosslinking polylysine with a bioreducible crosslinking agent increased the stability of polyplexes, and after masking the surface with PEG, a 10-fold increased *in vivo* plasma circulation following intravenous administration to mice, indicated that the disulfide bridges were capable of endowing good stability to the complex extracellularly, efficiently reducing within the cytoplasm or nucleus, and enabled transcription of DNA to contain in the vector, while the disulfide bonds were breaked up after cellular uptake upon reaching the reducing environment of the cytosol, released the reduced polymers and DNA [70].

4.1.2 Thiol Modification on Polymers

Thiolated polymers in combination with reduced glutathione were shown to facilitate release of nucleic acids and improve the uptake of hydrophilic macromolecules. Thiol modification of chitosan displayed superior transfection efficiency than chitosan alone. After modification of chitosan with thioglycolic acid, intermolecular and intramolecular disulfide bonds formed in chitosan polyplexes by mercapto group oxidation [71]. Lee prepared thiolated chitosan by the reaction with thioglycolic acid. Thiolated chitosan condensed pDNA forming nanocomplexes exhibited significantly improved gene delivery potential *in vitro* as well as *in vivo*. The enhanced gene delivery of thiolated chitosan may be explained that thiolation of chitosan reduced the positive charge density and pDNA complexing capacity, resulting in more rapid pDNA release [72].

The construction of thiolated polyethylenimine complexed with DNA caged polyplexes with a reversible intracellular unpacking property improved stability and transfection by Wang [73]. The synthesized thiolated polyethylenimine displayed sufficient DNA condensation ability. The shell of thiopolyplexes was cross-linked to form a caged structure due to the oxidation of the thiol groups in air. Then in the intracellular environment, the disulfide bonds could be cleaved by the action of GSH to hopefully release the enclosed DNA to improve transfection efficiency.

4.1.3 Acid-Labile Polymers

The degradation of gene carrier is important to reduce the cytotoxicity of the complexes. The acid-labile PEI will be less cytotoxic, due to they could be degraded into nontoxic LMW PEI.

The acid labile PEI was relatively stable at physiological pH, which showed close transfection efficiency to 25 KDa PEI, but induced much less cytotoxicity than 25 KDa PEI(Fig. (**6**)). Free PEI might induce an immediate toxicity and PEI/DNA complexes in cellular processing could produce a delayed toxicity. The acid-labile PEI degraded rapidly in acidic endosome condition, producing less toxic low molecular weight PEI. After release of DNA, low molecular weight PEI again produced from acid-labile PEI might have lower toxicity than nondegradable PEI. Then rapid degradation of acid-labile PEI decreased the delayed toxicity [74]. Acid-labile acetal or ketal bond-bearing cationic polymers were recently developed by Knorr *et al.* [75,76]. These polymers could provide adequate stability for DNA polyplex formation leading to efficient gene transfer, but also sensitivity toward a slight decreased due to the hydrolysis of the acetal or ketal bond in the endosomal acidic environment, which caused these polymers degradation and thus improved biocompatibility. So the OEI-based acid-sensitive polymers could display better gene transfer ability compared with acid-stable controls.

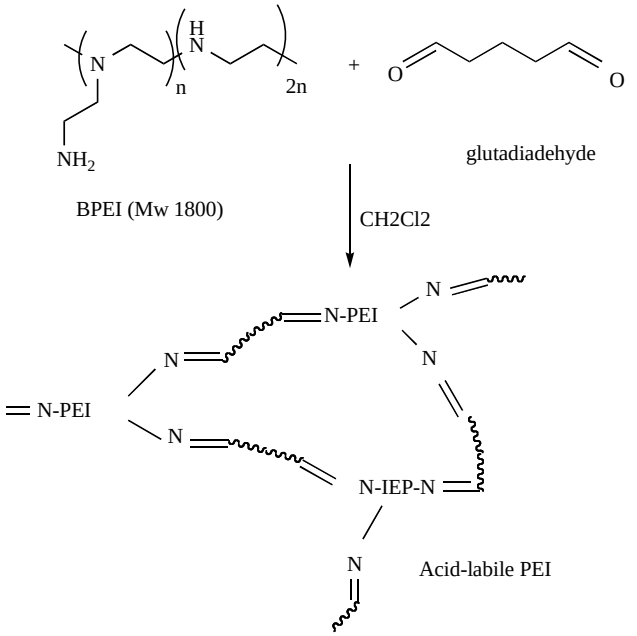

Fig. (6). Reaction scheme for the copolymerization of PEI and glutadialdehyde.

4.1.4 Hydrophobic Unit Modification

The hydrophobic unit in the polymeric carriers may assist dissociation of polymer/DNA complexes, to facilitate release of DNA, thus lead to higher transfection efficiency. For example, deoxycholic acid modified chitosan oligosaccharide (COSDs), due to their amphiphilic character formed core-shell type nanoparticle. Higher gene transfection by the deoxycholic acid conjugation might be explained to enhance gene condensation capacity of COSDs, hydrophobic moiety, deoxycholic acid had been originated by the increasing cell membrane-carrier interactions and destabilization of the cell membranes. Moreover, the detergent like amphipathic nature of deoxycholic acid could provide the COSDs with the facilitated bilayer permeabilizations. Membrane destabilization and buffering capacity of the chitosans originated by low pKa 6.0-6.5 resulted in enhancing gene delivery [77]. Stearic acid (SA), which grafted into the backbone of

chitosan oligosaccharide had also formed a core-shell structure of hydrophobic shorter graft chain of SA segments as an internal core and longer main chain cationic chitosan oligosaccharide (CSO) as a surrounding corona, which favored the escape of CSO-SA/DNA complex from endosome [78].

Alkylated chitosans (ACSs), self-aggregate in acetic acid solution, chitosan and ACS both can cause the fusion of dipalmitoylsn-glycero-3-phosphocholine (DPPC) multilamellar vesicles and the membrane destabilization. But, introducing alkyl side chains may result in a more evident alteration in the topological structure of DPPC. The transfection efficiency is raised and levels off after the number of carbons in side chain exceeds 8 upon lengthening the alkyl side chain. The higher transfection efficiency of the systerm by introducing alkyl side was presumably due to the increasing entry into cells facilitated hydrophobic interactions and easier unpacking of DNA from ACS vectors, that hydrophobicity induced weakening of electrostatic attractions between cargo and vectors [79]; furthermore, 5-β-cholanic acid modified glycol chitosan, where a hydrophobic moiety in the chitosan formed a hydrophobic domain inside the chitosan nanoparticle [80], galactosylated chitosans/DNA complex was taken up into cell via the receptor-mediated endocytosis pathway, and the transfection efficiency increased with the galactose density of complex increasing [81]. These chemical modificated chitosans had been widely studied to enhance transfection efficiency by increasing cell membrane–carrier interactions and endosomal escape.

PEI enables to condense DNA into nanometric particles suitable for *in vitro* and *in vivo* gene transfer applications. The lipophilicity-hydrophilicity balance of PEI plays a significant role in enhancing the gene transfection [48]. PEI was altered via the substitution of its primary amines with carboxylate-terminated short, moderate and long alkyl chains. Decreased DNA-binding ability and transfection efficiency resulted from increases in either the degree of substitution or hydrocarbon chain length [82,83]. The acylated PEI were 5-12 fold more efficient transfecting agents as compared to native PEI and commercially available transfecting agent lipofectin. Maybe acylation reduced the number of primary amines on the polymer and the surface charge, improving haemocompatibility and reducing cytotoxicity [84,85]. Dehshahri *et al.* investigated alkyate low toxicity, LMW 10 kDa branched PEI with a series of ω-bromoalkylcarboxylates with different chain lengths, followed by forming amide linkages between the terminal carboxylate moieties and oligoamines as spermine, spermidine, ethylendiamine and diethylentriamine. The hydrophobic modification of PEI is an effective strategy for improving transfection efficiency of polycation-based non-viral vectors while maintaining low toxicity [86].

4.1.5 Time-Dependent Self-Disassembly Strategy Improves Transfection Efficiency

To design cationic polymers which address one specific "late-stage" obstacle to polyplexes mediated transfection: the need for a cationic polymer to ultimately release DNA when it has reached a safe and suitable location within a cell, that is self-assembly and time-dependent self-disassembly strategy. When polyplexes have reached a safe and suitable location within a cell, cationic polymers should release or dissociate with DNA. Recently, Lynn *et al.* reported a new approach to achieve the prolonged release of DNA from multilayered films based on the fabrication of films using 'charge-shifting' cationic polymer (by fabricating films using polymers that undergo time-dependent changes in net charge) (Fig. (**7**)). This shift in net charge resulted in a weakening of the strength of electrostatic interactions between these polymers and DNA, thus promoted the release of DNA from polyplexes in solution. However, the backbone of polymer was not degradable and cleaved, but the hydrolysis of a single ester bond in the side chain reduced the net charge of a polymer chain by two through a degradable ester linkage. Hydrolysis of the side chains of polymer resulted in removal of cationic charge and introduces negative charge, finally produced a time-dependent'shift'in the net charge of the polymer [87].

The group also demonstrated that the addition of ester-functionalized, "charge-shifting" side chains to LPEI could be used to design polyamines that promote both self-assembly and time-dependent self-disassembly with DNA in defined physiological environments and demonstrated that the addition of "charge-shifting" side chains to LPEI could be used to increase levels of LPEI-mediated cell transfection significantly. The polymer functionalized with 20 mol % ester-functionalized side chains, mediated levels of transgene expression *in vitro* up to 8-fold higher than LPEI. The changes in the charge state of polymer, resulting from ester hydrolysis, could contribute to the destabilization of internalized polyplexes and to increase levels of transgene expression. The changes in the charge state of polymer could be understood in terms of time-dependent changes in the net charge of polymer, resulting from ester hydrolysis, could contribute to the destabilization of internalized polyplexes, promoted the disruption of polyplexes in intracellular environments effectively, thus to increase levels of transgene expression [88]. These 'charge-shifting' polymers and further modification present new opportunities to disrupt DNA-containing films under physiologically relevant conditions and design multilayered films that erode very slowly and release DNA over periods.

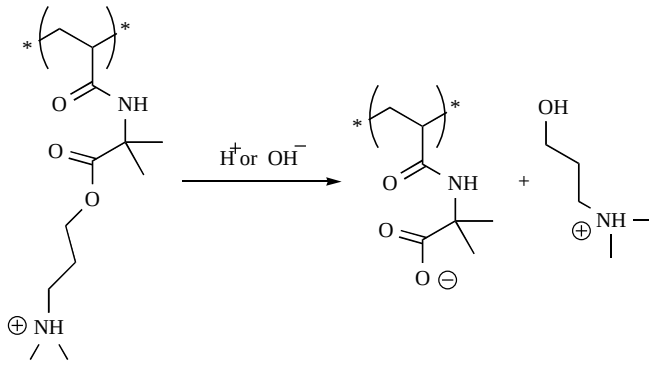

Fig. (7). Hydrolysis of a 'charge-shifting' cationic polymer.

4.2 Chemical Structural Modification on Polymers to Create Proton Sponge Effects

Chemical modificated polymers which are capable of creating proton sponge effects, could provide higher buffer

capacity and enhance endosomal escape then improve gene expression.

Concerning of the structure of PLL itself, chemical modification on PLL would overcome a series of disadvantages, resulted in enhanced transfection efficiency. One approach was to introduce histidine residues to PLL backbone, thus could create the desirable proton sponge effects similar to that of PEI polyplexes [89]. The histidine residues were shown to provide buffer capacity, thus to further enhance endosomal escape and *in vitro* gene expression [90]. For example, N-Ac-poly (L-histidine)-graft-PLL (PLH-g-PLL), was a PLL backbone with 25% of its ε-amine group's graft with PLH. The PLH profile was indicative of a neutral polymer at high pH and began to protonate at pH 6.5-5.0 and buffered the system through this range. Poly-L-histidine was used to induce membrane fusion at endosomal pH values in combination DNA polyplex forming properties of PLL and improved transfection efficiency over PLL [91].

Full deacylation of PEI affects nucleic acid transfection efficiency because that full deacylation of PEI possess much higher buffer capacity. Removal of the residual N-acyl moieties from commercial linear 25KDa PEI enhances its plasmid DNA delivery efficiency 21 times *in vitro*, as well as 10,000 times in mice with a concomitant 1,500-fold enhancement in lung specificity compared to native counterpart. Removal of the residual N-propionyl groups and full deacylation of PEI possess much higher buffer capacity that should ensure a more efficient endosomal escape of the polyplexes and, also the increaseing number of protonatable nitrogens could increase the binding affinity between the DNA and polymer and hence obtain a greater transfection efficiency [92]. Also, conjugation of acetate, butanoate, and hexanoate to branched PEI' primary amines at low degree of substitution (below 25%) resulted in moderate improvement of transfection activity as demonstrated by Putnam and colleagues [83].

The protonation of the PAMAM amines in weakly acidic conditions suppress the lowering of the pH in the endosomal/lysosomal compartment preventing degradation and rupturing of the endosome to release contents into the cytoplasm similar to PEI. Therefore, the buried tertiary amino groups of PAMAM also act as a proto-sponge in endosomes and enhance the release of DNA into the cytoplasm [33].

4.3 Chemical Modification on Polymer to Increase DNA Condensation Ability

As DNA itself is poorly taken up by cells, some required gene carriers can increase DNA condensation ability and facilitate the transport of DNA into the cell.

Given the promising transfection results of oligoethylenimine(OEI)-based crosslinked cationic polyesters, Russ *et al.* designed and prepared a serials of novel cationic polyesters, termed 'pseudodendrimers' [93]. The pseudodendrimers consisted of OEI in the center functionalized with an excess of degradable dioldiacrylates to form a pseudodendritic core that was subsequently modified on its surface with different oligoamines. The structure-activity followed that an increasing number of carbon atoms in the aliphatic spacer of the diacrylates was capable of increasing DNA condensation ability, showed that the increasing hydrophobicity of the pseudodendritic core played an important role in DNA binding, furthermore, the increasing nitrogen per coupled oligoamine on the pseudodendritic surface would result in stepwise rising ζ-potentials, which also led to enhanced DNA binding and condensation to nanoscaled polyplexes. The final products exhibited good biodegradability and low cytotoxicity with a half-life of 3 days under physiological pH conditions.

5. PEGYLATION

PEGylation means that the cationic polymers are modified with polyethylene glycol (PEG), which often can improve the solubility of the complexes, minimize their aggregation, reduce their interaction with proteins in the physiological fluid, finally, produce a shielding effect that counteracts effective DNA complexation and sterically stabilization to gene vectors' surfaces to improve transfection efficiency. Such as PEG-graft-trimethyl chitosan/DNA complexe led to improved colloidal stability of polyplexes and significantly increased cellular uptake, resulting in a significant, up to 10-fold increase of transfection efficiency in NIH/3T3, L929 and MeWo cells compared to trimethyl chitosan [94].

Linear PEG is a rather common biocompatible shielding reagent, widely used for gene delivery. Strong hydration and high conformational flexibility provide PEG with the steric stabilization. The PEGylation of polyplexes has been achieved either by condensing DNA with copolymers (pre-PEGylation) where PEG reagents containing maleimido groups used for coupling to mercapto-modified polycations before polyplex formation or coupling a PEG layer onto the surface of preformed polyplexes (post-PEGylation) [95]. PEGlyation of block copolymers used as polyplexes have been intensely investigated and have demonstrated significant advantages over non-PEGlyated macromolecules, not only in terms of reduce cytotoxicity *in vitro* and *in vivo* but also increase water solubility of polymer/DNA complex, decrease opsonisation processes due to the inherent steric barrier.

5.1 Effects of PEG Molecular Weight and PEGylation Degree

Relatively low molecular weight, low degree of substitution of PEGylation has provided polyplexes higher transfection efficiency.

The impact factors to polyplexes of PEG molecular weight: PEI-grafted PEGs (PEI-g-PEG) with more than 5 KDa PEG were synthesized to diminish the cytotoxicity and aggregation of PEI because the surface charge of polyplexes reduced due to the charge shielding effect of PEG [96]. Martin *et al.* synthesized a series of copolymers of cationic PEI and PEG. It showed that the PEG molecular weight was the main determinant of polyplex size, through its influence on aggregation. When grafted with PEG5000, both low and high molecular weight PEI-based copolymers formed small polyplexes, with minimal aggregation. Alternatively, when grafted with PEG550, both low and high molecular weight PEI-based copolymers formed complexes with oligonucleotides that appeared as much larger aggregates. But the

stability of polyplexes depended on level of the molecular weight of PEI and PEG grafting. Low molecular weight PEI2000-based copolymers formed extremely stable polyplexes that did not dissociate even at the highest polyanion concentrations [97].

A very low degree of substitution was capable of increasing transfection efficiency *in vivo*, while higher degrees of PEGylation offered high stability of PEI DNA complexes in a variety of solutions, however, usually came together with reduced transfection activities, probably because of hindering effects of PEG on the interaction of the complexes with the cells [98]. Petersen *et al*. studied the influence of PEI-g-PEG copolymer block structure on DNA complexation and biological activities as gene delivery system. PEI (25 KDa) was grafted to different degrees of substitution with PEG (5 KDa), it showed that with increasing degree of PEG grafting, complexation of DNA was impeded and complexes lost their spherical shape. Besides copolymers with many short PEG blocks formed large and diffuse complexes of high positive surface charge. Copolymers with only a few but long PEG blocked self-assembled to small and compacted condensates of low surface charge. Copolymers with many long PEG blocks generated complexes of ill-defined shape and of almost no surface charge. On the other, the molecular weight of PEG varied from 550 Da to 20 KDa was also investigated, that showed combination of large particles, low toxicity, and high positive surface charge as in the case of copolymers with many PEG 550 Da blocks proved to be most efficient for *in vitro* gene transfer [99].

High molecular weight PLL could increase DNA condensation ability and transfection efficiency and possess potential properties suitable as gene vector, but the PLL/DNA complexes showed a relatively high cytotoxicity and a tendency to aggregate and precipitate depending on the ionic strength [41,100] To solve this problem, PEGylation which could stabilize the PLL/DNA complexes is necessary. The fraction of PEG affected condensation between PLL and DNA, and the condensation was most efficient when the fraction of PEG in linear PLL was less than 60% of the polymer molecular weight. For dendritic PLL, condensation with DNA was also weak at high PEG contents, while for grafted PLL, the fraction of PEG showed no effect on DNA condensation [101]. In most case, linear polylysines were more efficient than most dendritic ones in terms of forming DNA polyplexes, and this might due to the more flexibility of linear and grafted PLL molecrles than dendritic ones, thus DNA could easy bind to the cationic charge groups in spite of PEG.

5.2 PEGylation to Reduce the Effects of Endosomal Escape

Endosomal escape might be limiting for PEG-shielded polyplexes of small size. Both the small size of targeted polyplexes and the stable PEG shield are known to be disadvantageous for endosomal release, but small shielded formulations are disadvantages for endosomal escape but indispensable for efficiency gene delivery. Wagner *et al.* had engineered epidermal growth factor receptor (EGFR) *via* PEG spacer covalently attached to PEI formed PEG-shielded targeted polyplexes. The polyplexes contained PEG to prevent undesired interactions with non-target cells and the PEG shield enable prolong blood circulation. However, the specifity of EGF polyplexes binding to the target cells and after the cell association, endosomal escape and nuclear transfection might be bottlenecks in the transfection process. So the group further exploited strategies to overcome the lower efficiency with respect to low endosomal release. PEG was incorporated into polyplexes in a bioreversible fashion to improve EGFR-targeted shielded polyplexes, resulting in endosomal removal of the PEG shield and re-activation of the endosomal escape activity [102].

The pH-sensitive cationic polymers conjugate PEG systems can significantly enhance gene transfection. The endosomal pH-sensitive cationic polymers for complex DNA through the conjugation of PEG using hydrazone and acetal based linkages, resulted in a significant improvement in the gene expression *in vitro* and *in vivo* when compared to the stably shielded polyplexes. The shielded polyplexes that could undergo a deshielding process were able to reach the transfection efficiency of the larger targeted polyplexes without shielding conjugate. The shielded polyplexe were stable at pH 7.4 for the transfection, the transfer efficiency of the reversibly shielded polyplexes are higher than the nonreversibly is likely due to variations in cell trafficking efficiency such as endosomal escape [103]. A novel acetal-based PEGylation reagent (PEG-acetal-MAL) was introduced by maleimide moiety for pH-sensitive conjugation of PEG to thiol-functionalized biomolecules. PEG-acetal-MAL was conjugated to mercapto-modified PEI for reversible shielding of polyplexes, which showed proper and persistent shielding at physiological pH but at the same time were deshielded quickly at the acidic pH 5. The pH-sensitive and reversibly shielded (PEG-A-PEI) polyplexes were found to have approximately 10-fold enhanced gene transfer efficiency than stable shielded (PEG-S-PEI) polyplexes when tested on Renca-EGFR cells and K562 cells lines [104].

5.3 PEGylated Polymers for Targeting

Targeting modification can greatly enchance gene transfection efficiency. Targeting modifications which could be introduced targeting ligands may result in increased gene expression and allow to direct transfection complexes more specifically to selected cell types and reduce undesired side-effects in non-target cells at the same time. Usually, the PEG corona on the complexes could provide a linker molecule for the decoration of the complexes with a targeting ligand. The role of the linker might not be limited to the coupling of the ligand and could be that of a spacer to enhance the access of the ligand to its target receptor. This may be the assumption that the observed steric repulsion through the PEGylated surface of complexes might repel opsonising proteins and restrict ligand-receptor recognition [105].

Depending on the type of ligands, modification of polyplexes surface with appropriate amount of PEG did not block ligand mediated internalization [106]. Muller *et al.* studied with microspheres coated with a PLL-g-PEG copolymer that was terminally decorated with biotin, PEG was used stealth corona and spacer molecule. While PEG (2 KDa) was sufficient to ensure stealth function, PEG (3.4 KDa) was used for the coupling of the ligand to ensure its accessibility. Accessibility of the biotin ligand was demonstrated with fluorescently labelled streptavidin, and it showed increasing

ligand densities could lead to increasing streptavidin binding [107]. RGD-targeted PEI polyplexes had shown enhanced gene transfer levels compared to PEI alone. However, coupling RGD via a PEG spacer to PEI, targeting was partially reduced, possibly due to hide of the RGD sequence inside the PEG cloud [108,109]. Suh *et al.* demonstrated the importance of optimal composition of RGD-PEG-PEI conjugates, showed that transfection efficiency decreased as the degree of PEG-RGD grafting onto PEI increased [109].

5.4 The Application and Development of PIC

The term 'polymeric micelles' (PIC) which result from cooperative electrostatic interactions between the genetic material and a cationic copolymer presenting a water-soluble nonionic segment, associates in solution phase with definite size and appreciable stability. The charge with PIC compensated nucleic acid/cationic chains self-assemble into a micellar core while the hydrophilic segments form a protecting corona which not only confers solubility and colloidal stability to the system but also shields any cationic charges [110].

PIC micelles demonstrated remarkable properties as delivery vehicles for DNA, excellent colloidal stability in protein aceous media, high solubility in aqueous media, high tolerability toward nuclease degradation, and minimal interaction with biological components, including proteins and cells, and prolong blood circulation compared to the other conventional polyplex and lipoplex systems [111,112].

A core-shell-type polyion complex micelle with a disulfide cross-linked core was prepared through the assembly of iminothiolane-modified poly (ethyleneglycol)-block-poly (L-lysine) [PEG-b-(PLL-IM)] and siRNA. The cleavage of disulfide cross-links exhibited remarkable stability in physiological medium, underwent dissolution upon the reduction of disulfide cross-links to allow the release of entrapped siRNA, and achieved 100 times higher transfection efficiency than PIC assemblies without disulfide cross-links [29].

Furthermore, pH-sensitive PEGylation PIC colud be used as targetable and low invasive endosomolytic agents to induce the enhanced transfection efficiency of nonviral gene vectors. PIC was obtained following the electrostatic complexation of pH-sensitive methacrylic acid (MAA) copolymer, a model oligodeoxynucleotide (ODN), and a cationic lipid, intimately integrated within the structure of neutral, water-soluble, and well-defined PIC. Upon a decrease in pH, the resulting PIC partially dissociated to release membraneactive fragments that efficiently destabilize lipid bilayers [113]. Oishi *et al.* designed and prepared a novel ABC triblock copolymer for constructing a pH-responsive and targetable gene vector. The copolymer, lactosylated poly(ethyleneglycol)-block-poly(silamine)-block-poly[2-(N,N-dimethylamino)ethyl methacrylate] (Lac-PEG-PSAO-PAMA), consisted of lactosylated poly(ethylene glycol) (A-segment), a pH-responsive polyamine segment (B-segment), and a DNA-condensing polyamine segment (C-segment). The Lac-PEG-PSAO-PAMA spontaneously associated with pDNA to form three-layered polyplex micelles with a PAMA/pDNA PIC core, an uncomplexed PSAO inner shell, and a lactosylated PEG outer shell. The micelles exhibited a specific cellular uptake into HuH-7 cells (hepatocytes) through asialoglycoprotein (ASGP) receptor-mediated endocytosis and achieved a far more efficient transfection ability of a reporter gene compared to the Lac-PEG-PSAO/pDNA and Lac-PEG-PAMA/pDNA polyplex micelles composed of the diblock copolymers and pDNA [112].

6. COMBINATION STRATEGIES

The classical synthetic routes of the block and graft copolymers composed of cationic moiety and nonionic-hydrophilic moiety can be categorized into two methods: polymerization of cationic moiety from the end of the hydrophilic moiety and the coupling reaction of two moieties [114]. The advantages of combination strategies with different polymers are obvious that possess defined molecular weight, and topology and introduction of hydrophilic and lipophilic groups and synthesis of simple or composited different long chain block or graft copolymers, also further better understand structure-activity relationship based on the sequence-defined polymers. As the improved material industry and synthetic chemistry for optimize polymers with uniform size, topology and transport domains, the existing

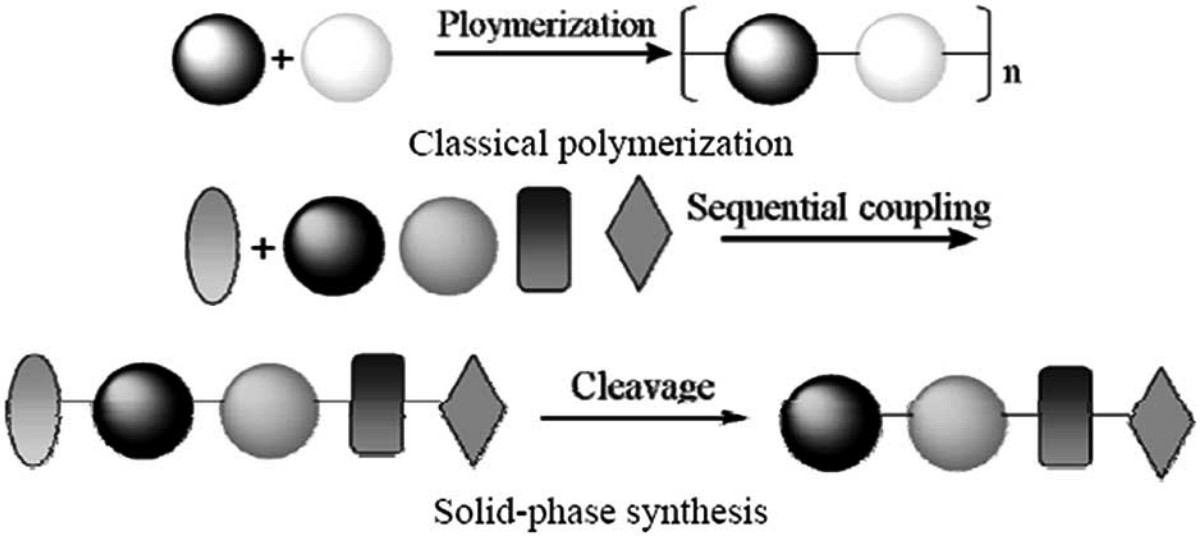

Fig. (8). Schematic representations of classical polymerization and solid-phase methodology.

experience from gene delivery boosts rapid development of polymeric carriers. Synthetic control over block copolymer chemistry enables tunable design of copolymer material properties. Solidphase supported synthesis allows the sequence-specific synthesis of monodisperse polymers with absolute control of position and composition of every monomer by sequential elongation of the polymeric chain [11].

The synthetic nature of copolymers opens new options, such as synthesizing multiblock copolymer (MBC) of the type (AB)n or (ABC)n, amphiphilic block copolymers, higher stability but biodegradable, bioresponsive, multi-group modification even different molecular weight and different linkage modes of copolymers, so the ideal cationic polymers desired can be applied in gene delivery.

Combination of PEI and chitosan could induce high gene transfection efficiency and low cytotoxicity [115,116]. Lu *et al.* prepared N-maleated chitosans (NMC) grafted PEI (800 Da) copolymers by Michael addition. After the introduction of low molecular weight PEI, all the copolymers exhibited improved solubility and buffering capacity, NMC_{5K}-*g*-PEI and NMC_{10K}-*g*-PEI showed comparable transfection activity and lower cytotoxicity as compared with PEI (25 KDa) in both 293T and HeLa cells, whereas NMC_{50K}-*g*-PEI showed higher cytotoxicity and lower transfection activity, NMC_{50K}-*g*-PEI with high molecular weight could much easily entangle free DNA through electrostatic interactions, and the strong interactions between DNA and the copolymer resulted in highly stable complexes, thereby preventing dissociation within the cell and ultimately precluding the translation of DNA [116].

Biodegradable PLGA and PLA conjugated cationic polymers PEI, PLL might increase the encapsulation efficiency, modulate the release kinetics, and improve the transfection ability of gene material [117,118]. Patile has investigated PEI was incorporated in the PLGA matrix to improve siRNA encapsulation and release. It was found that inclusion of PEI in the nanoparticle matrix led to greater cellular accumulation of complexes, but PLGA-PEI nanoparticles were taken up more than PLGA complexes only, the mild cationic charge of PLGA-PEI in the acidic pH of the endo-lysosomal compartment might enable a greater fraction of the nanoparticles to escape the lysosomes [118].

7. MULTIFUNCTIONAL POLYPLEXES

Multifuntional modification platform is a hot topic of gene transfer system based on the cationic polymers formed polyplexes. The assembly of multifunctional gene transfer systems is designed in order to take advantages of viral vectors properties such as their small size (≤100 nm), capability to escape the immune system sentinels, cell tropism, cytoplasm delivery and/or nuclear targeting and penetration [119].

7.1 Multilayered System Design

The multilayered systems are designed by systematic step-by-step de-layering. The layer-by-layer assembly of multilayered system takes advantage of attractive electrostatic forces between oppositely charged polymers and are, in general, technically straight forward to implement: the iterative dipping of an object into two different solutions of oppositely charged polyelectrolytes yields multilayered films composed of alternating layers of cationic and anionic polymers [120].

Multilayered system contained targeting, biosensing and gene delivery molecules that were released a layer at a time. This produced a smart system that resulted in an ordered series of molecular programming relatively. Core materials of the most interior were the first step in creating a multifunctional layered system. Then molecular layers were attached around the core material. Therapeutic genes could be tethered to the surface of these core particles in a manner such that these molecules were free to interact with their eventual targets. The middle functional layer(s) included intracellular targeting molecule such as aptamers, antibodies, peptides, and other molecules. Lastly, the outermost layer of the nanomedical system contained the cell surface targeting molecules designed to help the NP bind to the cell of interest and to try to avoid binding to other cell types [121].

7.2 Multicompent Coupling Strategies

These multicompent coupling stategies with cationic polymers share three main design components: platform (core) material, encapsulated payload/biologically active agents, and targeting/surface properties. In this system, long-circulating PEG chains, diagnose agent, therapeutic agent, therapeutic nucleic acid construct, targeting moiety and imaging modality are incorporated multifunctional nanosystems to overcome biological barriers and enhance transport across biological barriers and drug availability and residence at the tumor site, so as to enhance intracellular uptake, to achieve diagnose, therapy, imaging tracing integration [122,123].

Moffatt *et al.* reported a novel coupling strategy involving salicylhydroxamic acid and phenyl (di)boronic acid molecules to attach the CNGRC peptide to PEI/DNA for CD13 targeting in tumors. The targeting polyplex, CNGRC/PEG/PEI/DNA-p53/

NLS/DNTS, with EBV-based episomal vector for sustained p53 gene expression, the multicomponent vector also co-targeted tumor and tumor-associated endothelial cells but not normal cells, and had more efficient therapeutic index than each vector administered as a single modality. The use of an efficient coupling strategy without compromising the vector's integrity for DNA condensation and endosomal escape, nuclear import, tumor-specific and persistent p53 gene expression clearly provided a basis for developing a single combinatorial approach for non-viral gene therapy [124].

Zdravka *et al.* established a feasibility of a novel technology centered around multifunctional magnetic nanocarriers, which concurrently delivered siRNA to intact pancreatic islets and could be detected by magnetic resonance and optical imaging, demonstrated after *in vitro* incubation, magnetic nanoparticles carrying siRNA designed to target the model gene for enhanced green fluorescent protein were efficiently taken up by murine pancreatic islets, derived from EGFP transgenic animals [125].

7.3 Polymersomes

Polymersomes, are also used as a multifunctional polymer vesicles which self-assembled from a diverse array of synthetic amphiphilic block copolymers containing hydrophilic and hydrophobic blocks [126,127]. For example, poly (ethyl ethylene)-b-poly (ethylene glycol) (PEE-PEG), poly (lactic acid)-b-poly (ethylene glycol) (PLA-PEG), PEG is a common choice as the hydrophilic block of copolymers that self-assemble into vesicles. PEG is used to anchor these moieties such as antibodies, or RGD-containing peptides. Polymersomes have been shown to possess superior biomaterial properties, including greater stability and storage capabilities [128,129], as well as prolonged circulation time, as compared to liposomes (vesicles derived from phospholipids). Polymersomes could simultaneously encapsulate hydrophilic components in their aqueous interiorand hydrophobic molecules within their thick lamellar membranes. Besides, biologically active ligands, such as antibodies, can be readily conjugated to the exterior brush surface to target the vesicles and to provide a therapeutic response [130].

Because of the large compartment for carrying hydrophilic payload and the membrane for hydrophobic substances, polymersomes are very promising in biomedical and biotechnological applications. Polymersomes could enhance bioavailability and cellular targeting and delivery genes. Moreover, amphiphilic copolymers as building blocks had low critical aggregation concentrations and very slow chain exchange dynamics, so they had very slow rates of dissociations and allowed the retention of the payload for long periods depending on the properties of the hydrophobic block of the copolymer [131]. These properties of the polymersomes architecture effectively create a multimodal platform, which could be used for therapeutic, drug delivery or diagnostic and imaging applications.

8. OUTLOOK

Cationic polymers are valuable tools for gene delivery and therapy. This view is about that the rational design of polyplexes with clearly defined structure-function relationships, then facilitate the development of gene transfer. The first polymeric gene carriers, including PEI and polylysine derivatives, have already been tested in clinical trials, focusing on local administration to tumors (PEI in bladder carcinoma), or regional delivery to airway epithelium (PEG-polylysine in cystic fibrosis) [11]. Besides, Dharmacon company thrusted out DharmaFECT transfection reagents that are capable of high cotranfection siRNA and plamid under the condition of maintaing high cell viability.

Further optimization on synthetic cationic polymers to improve stability, biodegradable, bioresponsive, enhance gene transfection but decrease the cytotoxicity, depends on the improved chemistry and material industry, further identify of transfection mechanism *in vivo*, influence factors and sequence-defined structure-activity relationships. However, gene transfection efficiency of cationic polymers is not comparable to viral vectors, though cationic polymers spontaneously self-assemble with nucleic acid into nano-sized complexes with morphologies that often resemble viral particles. The ease of chemistry has facilitated the development of cationic vectors and the production scale of non-viral vectors also exceeds the viral vectors production. But the future development of cationic polymers is towards "artificial virus", which possess both virus and non-virus merit with higher transfection efficiency of virus and various kinds of non-virus properties. Such as bacterial magnetic particles (BMPs)-PEI was used a novel and efficient non-viral gene delivery system which displayed a high transfection efficiency and low toxicity both *in vitro* and *in vivo* [132]. Further strategies to optimize synthetic vectors that is the "artificial virus" approach, whereby various delivery functions are segregated into separate modules, targeting and shielding molecules have been incorporated into the carrier formulations for enhanced and more specific performance, as is the case with native viruses [133]. However, several aspects, for example, bioresponsive strategies using acidic environments, the hydrolysis of polymers or biodegradable polymers based on rational chemical modification, proper assembly of multifunctional modification, and also build larger supramolecular structures such as cyclodextrin-based supramolecular architectures [134] are considered when rational design polyplexes.

Together with the better understanding structure-function relationships with transfection efficiency and development of polymer itself and multifunction mofication on the surface of polymer, these will contribute to the design of polyplexes, which will fuel clinical development pipelines in the near future.

ABBREVIATIONS

ACSs	=	Alkylated chitosans
ASGP	=	Asialoglycoprotein
BMPs	=	Bacterial magnetic particles
COSDs	=	Deoxycholic acid modified chitosan oligosaccharide
CSO	=	Chitosan oligosaccharide
DPPC	=	Dipalmitoylsn-glycero-3-phosphocholine
DSP	=	Dithiobis (succinimidylpropionate)
DTBP	=	Dimethyl 3,3V-dithiobispropionimidate
EGFR	=	Epidermal growth factor receptor
HD	=	Hexanediol diacrylate
HMW	=	High molecular weight
Lac-PEG-PSAO-PAMA	=	Lactosylated poly(ethyleneglycol)-block-poly(silamine)-block-poly[2-(N,N-dimethylamino)ethyl methacrylate])
LMW	=	Low molecular weight
MAA	=	Methacrylic acid
MBC	=	Multiblock copolymer
MPS	=	Mononuclear phagocyte system
MW	=	Molecular weight
NMC	=	N-maleated chitosans
ODN	=	Oligodeoxynucleotide
OEI	=	Oligoethylenimine

PAMAM = Poly (amido amine)

PEE-PEG = Poly (ethyl ethylene)-b-poly (ethylene glycol)

PEG = Polyethylene glycol

PEG-b-(PLL-IM) = Iminothiolane-modified poly (ethyleneglycol)-block-poly (L-lysine)

PEI = Polyethylenimine

PEG-b-PBLG = Poly(ethylene glycol)-blockpoly(γ-benzyl L-glutamate)

PIC = Polymeric micelles

PLA-PEG = Poly (lactic acid)-b-poly (ethylene glycol)

PLH-g-PLL = N-Ac-poly (L-histidine)-graft-PLL

PLL = Poly (L-lysin)

TMC = N,N,N-trimethylated chitosan

TMO = N,N,N-trimethylated chitosan oligomer

SA = Stearic acid

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Received: October 10, 2009 Revised: December 05, 2009 Accepted: December 05, 2009