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Polymeric Carriers for Gene Delivery: Chitosan and Poly(amidoamine) Dendrimers

Qingxing Xu^{1,2}, Chi-Hwa Wang², and Daniel Wayne Pack^{1,*}

¹Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, 600 S. Mathews Avenue, Urbana, IL 61801, USA

²Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore 117576

Abstract

Gene therapy is a potential medical solution that promises new treatments and may hold the cure for many different types of diseases and disorders of the human race. However, gene therapy is still a growing medical field and the technology is still in its infancy. The main challenge for gene therapy is to find safe and effective vectors that are able to deliver genes to the specific cells and get them to express inside the cells. Due to safety concerns, synthetic delivery systems, rather than viral vectors, are preferred for gene delivery and significant efforts have been focused on the development of this field. However, we are faced with problems like low gene transfer efficiency, cytotoxicity and lack of cell-targeting capability for these synthetic delivery systems. Over the years, we have seen a variety of new and effective polymers which have been designed and synthesized specifically for gene delivery. Moreover, various strategies that aimed at enhancing their physicochemical properties, improving transfection efficiency, reducing cytotoxicity as well as incorporating functional groups that offer better targetability and higher cellular uptake are established. Here, we look at two potential polymeric carriers, chitosan and poly(amidoamine) dendrimers, which have been widely reported for gene delivery. For chitosan, the interest arises from their availability, excellent non-cytotoxicity profile, biodegradability and ease of modification. For poly(amidoamine) dendrimers, the interest arises from their ease of synthesis with controlled structure and size, minimal cytotoxicity, biodegradability and high transfection efficiencies. The latest developments on these polymers for gene delivery will be the main focus of this article.

Keywords

chitosan; poly(amidoamine); polymer; carrier; cationic; gene; transfection; ligand

1. INTRODUCTION

Over the past decades, gene therapy has been heavily investigated. With the rapid advancement in molecular biology and biotechnology, the chromosomal defects for a large number of human hereditary diseases have been identified and they significantly raise the prospects for cures using gene therapy. Since the first clinical trial of gene therapy was conducted for the treatment of severe combined immunodeficiency (SCID) in 1990 [1], a number of successes have been reported in recent years. In 2006, Ott et al. [2] reported promising results in the treatment of chronic granulomatous diseases (CGD) under nonmyeloablative conditioning. Previous attempts in correcting human CGD by gene

*Correspondence should be addressed to: Daniel Wayne Pack, Tel: (217) 244–2816, Fax: (217) 333–5052, dpack@illinois.edu.

therapy in the absence of bone marrow ablation have been unsuccessful. The patients had X-linked CGD (X-CGD), a primary immunodeficiency caused by mutations in the gp91^{phox} gene, and were treated with genetically modified cells. Substantial engraftment of gene-transduced cells was observed and superoxide production was at therapeutic level that was crucial for immunity restoration. In the same year, Morgan et al. [3] reported success in the treatment of advanced metastatic melanoma. The patients were treated with genetically engineered lymphocytes and results showed sustained cancer regression. In 2008, Bainbridge et al. [4] reported positive results for the world's first gene therapy for inherited blindness. Patients with Leber's congenital amaurosis (LCA), a rare inherited eye disease caused by an abnormality in the RPE65 gene, were treated for vision improvement. One patient showed significant improvement in visual function on microperimetry and dark-adapted perimetry, and in a subjective test of visual mobility. Though there have been many clinical trials for gene therapy being approved worldwide, the small number of success that has been reported so far is disappointing. While the concept of gene therapy appears to be simple, there are a number of obstacles which need to be overcome in practice [5].

Gene delivery systems can be divided into two categories: viral and non-viral vectors. Viral vectors such as retrovirus, lentivirus, adenovirus and adeno-associated virus, are typically very efficient in gene transfer. They can be transformed into effective gene delivery systems by replacing part of their genome with a therapeutic gene. However, safety concerns remain the main bottleneck to the clinical application of viral gene delivery. Retroviruses, for instance, suffer from oncogenic risks associated with insertional mutagenesis, leading to the development of malignant cells. Moreover, viral vectors may also elicit a series of inflammatory or immune responses as a result from repeated administration. Besides, the packaging capacity of viral vectors is limited and current production methods are difficult to scale up to manufacture relevant quantities for commercial and clinical applications. Non-viral vectors, on the other hand, have captured significant interest due to their improved safety, greater flexibility and ease of synthesis. They consist of complexes formed by either cationic lipids or cationic polymers, which are able to protect DNA from nuclease degradation and deliver them to the cells. However, complexes formed by cationic liposomes, also known as lipoplexes, have several limitations in gene delivery. They include difficulty in reproducibly fabricating liposomes and lipoplexes, toxicity in vitro and in vivo, and colloidal stability, especially upon systemic administration [6]. Thus, non-viral vectors based on cationic polymers or complexes formed by cationic polymers, also known as polyplexes, are being researched extensively for gene delivery [7]. Most of the researches on cationic polymers focus on addressing problems associated with low transfection efficiency, cytotoxicity and non-specific cellular uptake.

The gene transfer efficiency of non-viral vectors has generally been unsatisfactory. They often lack one or more critical functions needed to overcome the series of extracellular and intracellular obstacles during gene delivery [8]. An ideal carrier should protect the DNA from nuclease degradation and be stable upon administration into the biological environment. The carrier should have targeting moieties to allow cell-targeted delivery and enhance cellular uptake. Upon internalization, the carrier system must escape from the endocytotic pathway and be transported towards the nucleus. At some point during the process, the carrier must release the DNA so that they can be transcribed in the nucleus. Thus, the understanding of these obstacles is critical in the design of new carriers for gene delivery. In this article, we aim to discuss and review some of the recent developments on chitosan and poly(amidoamine) (PAMAM) dendrimers based formulations for gene delivery. These two non-viral vectors have gained significant interest as gene delivery systems.

2. CHITOSAN

Chitosan is a linear polysaccharide which consists of randomly distributed 2-N-acetyl-2-deoxy-glucose (N-acetyl-glucosamine) and 2-amino-2-deoxy-glucose (glucosamine) residues with β -1,4-linkage as shown in Fig. (1). Chitosan is a product derived from alkaline N-deacetylation of chitin from which it can be obtained via treating shells of crustaceans with dilute hydrochloric acid to remove minerals, treating them with sodium hydroxide solution to remove protein and then treating them with potassium permanganate solution for decolorization [9]. The primary amine of chitosan has a pKa value of about 6.5 [10]. Thus, chitosan is soluble in a variety of acids, but insoluble at neutral and alkaline pH values. Besides pH, the solubility of chitosan is also significantly influenced by the degree of deacetylation, molecular weight and ionic strength of the solution. To date, chitosan has been widely used as a carrier for drug and gene delivery in various applications [11].

Chitosan is one of the most reported non-viral naturally-derived cationic gene polymers, due to its biodegradability, biocompatibility, non-allergenicity, mucoadhesive property and strong affinity with DNA. Chitosan has been shown to have low cytotoxicity in a series of tests performed on both experimental animals [12] and humans [13]. Its excellent soft tissue compatibility has also been demonstrated. It is found that the zeta potential of chitosan with 100% deacetylation is not highly positive, but very close to zero [14]. This suggests that chitosan is so bio-inert that it may not give any strong stimulus to the surrounding tissue. Thus, it has been widely used for various artificial skin [15], tissue reconstruction [16] and wound healing bandage [17] applications. The cationic chitosan can interact strongly with the negatively charged mucosal surface, leading to useful mucoadhesive property [18–20]. Many of the applications involve using chitosan for nasal and peroral delivery of peptide drugs in order to improve drug permeation and absorption [21–27]. At acidic pH values, chitosan has been shown to effectively bind DNA via electrostatic interactions and protect them from nuclease degradation [28]. At neutral or alkaline conditions, in which chitosan is slightly charged, non-electrostatic interactions, such as hydrogen bonding and hydrophobic interactions could be responsible for the binding between chitosan and DNA [29].

Multiple studies have shown that the binding affinity of chitosan for DNA, the stability and the transfection efficiency of chitosan-DNA complex are dependent on several parameters, including: i) molecular weight [30–36], ii) degree of deacetylation [33, 34, 37], iii) nitrogen to phosphate charge ratio (N/P) [30–33, 35, 38], iv) plasmid concentration [30, 32, 35, 36], v) serum concentration [30, 32, 35, 38], vi) pH of transfection medium [30, 31, 33, 35, 36], and so on. The variation of these parameters can significantly influence the cellular uptake and endosomal escape of the complex, and subsequently, the gene expression. Nevertheless, in comparison with polyethylenimine (PEI), which generally shows high transfection efficiency, chitosan has a lower efficiency. The effectiveness of PEI has been attributed to its high buffering capacity, especially at the acidic pH found in the endosomal vesicles, which leads to trapping of positive ions by the amines (proton sponge effect), followed by subsequent endosomal rupture and escape into the cytoplasm [39]. The difference in the buffering capacities of PEI and chitosan has been confirmed from acid/base titration [39]. In particular, PEI shows a high buffering capacity over a wide range of pH values. In contrast, the buffering capacity of chitosan is modest and is restricted to the pH interval between 5 and 7.

Over the years, chitosan has been widely investigated for a number of biomedical and pharmaceutical applications. Despite its desirable properties such as biodegradability, biocompatibility and low cytotoxicity, chitosan still lacks cell-targeting ability and exhibits low transfection efficiency for gene delivery. These problems are impeding the progress of chitosan as a potential gene carrier. In the following sections, various derivatives of chitosan

are discussed, all of which are aimed at improving chitosan as a gene carrier and enhancing its transfection efficiency both in vitro and in vivo. Selected examples of chitosan derivatives are shown in Fig. (2).

2.1. Hydrophilic Modification

Chitosan has captured significant attention due to its excellent characteristic as a mucoadhesive polymer. Chitosan in its protonated form is able to interact with the epithelial tight junctions and activate the openings so as to allow permeation of hydrophilic molecules [40]. In particular, chitosan can bind strongly to the intestinal epithelium, inducing redistribution of F-actin and tight junction protein ZO-1 [41]. However, at pH above its pKa value, chitosan has limited solubility and its penetration activity is significantly reduced [41]. These are major drawbacks for its potential use under physiological conditions. Thus, there is a need for chitosan derivatives with increased solubility, especially at neutral and basic pH values.

2.1.1. Trimethyl Chitosan—Trimethyl chitosan (TMC), in which a fraction of the amines are quaternized providing a permanent positive charge, is a useful derivative of chitosan with excellent solubility and basicity as compared to native chitosan and its salts. TMC, even with a low degree of quaternization, is soluble in a wide range of pH values and can be prepared with high concentrations [42]. Similar to the native chitosan, TMC exhibits mucoadhesive properties and is capable of opening tight junctions beyond a certain degree of quaternization [43–47]. TMC's desirable physicochemical properties under physiological conditions have led to its widespread use in drug and gene delivery for various biomedical and pharmaceutical applications [48–59]. TMC may be tailored to optimize the transepithelial delivery of drug molecules or the transfection efficiency of plasmid DNA.

TMC was first synthesized in the 1980s [60, 61] and can be prepared by reductive methylation of chitosan with methyl iodide at elevated temperature in a strong alkaline environment to bind the acid being generated during the reaction taking place and to avoid protonation of the unreacted primary amine groups. TMC with different degrees of quaternization can be prepared by i) adjusting the number of reaction steps [62–64], ii) adjusting the reaction time [62, 63], iii) using different bases [63, 64], or iv) using different degrees of deacetylation of chitosan [65]. Unfortunately, at higher degrees of quaternization, O-methylation on the 3- and 6-hydroxyl groups of chitosan has been reported and the products are less soluble. In order to synthesize O-methyl-free TMC, dimethylated chitosan is synthesized first using the Eschweiler-Clarke reaction with formaldehyde and formic acid, before it is converted into TMC using methyl iodide [66].

It is interesting to note that though mucoadhesive properties and cytotoxicity of TMC with different degrees of quaternization have been examined by many research groups, the observed results have been compared and reported to be controversial [42, 67]. In a study conducted by Sandri et al. [55], they obtained TMC from two grades of chitosan, 1460 and 580 kDa, with degrees of quaternization ranging from 4 to 90% and 3 to 78% respectively. The mucoadhesive and penetration properties towards buccal mucosa were observed to improve upon increasing the degree of quaternization of TMC. However, Snyman et al. [43] obtained TMC from chitosan of 148 kDa with degrees of quaternization ranging from 22 to 49% and found a decrease in the mucoadhesive properties with an increase in the quaternization of TMC. Florea et al. [57] reported that TMC was non-toxic even with high degrees of quaternization. However, Kean et al. [68] observed a general trend of increasing toxicity with increasing degree of quaternization.

Besides the degree of quaternization, the mucoadhesive properties and cytotoxicity of TMC may be dependent on two main factors [4], i) polymer chain flexibility during conformation

changes that arise from interactions between the positive charges on the TMC and the negative sialic groups of the glycoproteins in the mucus layer, and ii) steric effects due to the presence of attached methyl groups in shielding the positive charges on the TMC. Jintapattanakit et al. [67] shed some light on the discrepancy by investigating the influence of the degrees of quaternization and dimethylation on the mucoadhesive properties and cytotoxicity of TMC. The authors illustrated that the properties were dependent on the ratio between the degree of dimethylation and the degree of quaternization of the TMC. In particular, for the same degree of dimethylation of 40%, the mucoadhesive bond strength of TMC of 40% quaternization was 2-fold higher than that of 10% quaternization. In addition, for the same degree of quaternization of 20%, the mucoadhesive bond strength of TMC of 60% dimethylation was 3-fold lower than that of 20% dimethylation. The cytotoxicity of TMC decreased when the dimethylation to quaternization ratio was higher than 1 and TMC was non-toxic when the ratio was about 3:1. The data strongly suggest that the mucoadhesive properties and cytotoxicity of TMC are influenced by the degree of quaternization (positive charge density) and the degree of dimethylation (steric effects).

The degrees of quaternization, dimethylation and O-methylation affect the solubility of TMC. Jintapattanakit et al. [67] showed that the water solubility of TMC with a low degree of quaternization and a high degree of dimethylation decreased in a basic solution. Such polymers contained a large proportion of non-quaternized residues which led to a decrease in solubility. The authors observed that the degree of dimethylation greatly influenced the water solubility when the degree of quaternization was lower than 24%. In addition, the solubility of TMC with a high degree of quaternization was found to be independent of pH.

The physicochemical properties and cytotoxicity of O-methyl-free TMC are also investigated. Interestingly, Verheul et al. [66] found that O-methyl-free TMC with 24% quaternization was insoluble at pH 7 while TMC with 22% quaternization and degrees of O-methylation of 12 and 18% at the carbon-3 and carbon-6 positions respectively, was soluble. The authors explained that intra- and/or inter-molecular interactions may be dominant in TMC with low degrees of quaternization, and it resembled chitosan with low solubility. Thus, the presence of O-methylation might reduce such interactions, resulting in higher solubility. With higher degrees of quaternization, electrostatic repulsion among the positively charged groups becomes dominant, leading to a decrease in intra- and/or inter-molecular interactions and a higher solubility. However, high degrees of O-methylation decreased the solubility of TMC even with high degrees of quaternization. O-methyl-free TMC were found to enhance the opening of the tight junctions with increasing degree of quaternization. However, they induced significant toxic effects. Moreover, O-methyl-free TMC exhibited a greater decrease in cell viability than the corresponding O-methylated TMC.

The effect of the degree of quaternization of TMC on the transfection efficiency has been studied. Thanou et al. [48] investigated the complexation behavior of polyplexes formed by trimethylated chitosan oligomers (TMO) with 40% (TMO-40) and 50% (TMO-50) quaternization and evaluated their transfection efficiencies in COS-1 and Caco-2 cells. It was found that the size of the TMO-DNA complex was inversely proportional to the amount of oligomer added, and the TMO bound stronger to DNA than the unmodified chitosan oligomers. TMO were tested for their transfection efficiencies and compared to N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium sulphate (DOTAP) liposomes and chitosan oligomers. In particular, for the COS-1 cells, in the absence of serum, DOTAP lipoplex exhibited the highest transfection efficiency as compared to the naked DNA (control group). The transfection efficiency of TMO-DNA complex was significantly higher than that of chitosan oligomer-DNA complex; TMO-50 yielded efficiencies ranging from 5-fold (DNA/oligomer weight ratio of 1/6) to 52-fold (DNA/oligomer weight ratio of 1/14) while

TMO-40 yielded efficiencies ranging from 26-fold (DNA/oligomer weight ratio of 1/6) to 131-fold (DNA/oligomer weight ratio of 1/14). It is interesting to note that the effect of serum was minimal for the TMO-DNA complex but not for the DOTAP lipoplex. For the Caco-2 cells, the transfection efficiencies of DOTAP lipoplex and control group were similar. On the other hand, the TMO and chitosan oligomers mediated slightly higher transfection efficiencies than DOTAP, for which all the levels were significantly lower than those observed for the COS-1 cells. In another study, Germershaus et al. [69] conducted a similar study with the inclusion of polyethylene glycol (PEG) grafted TMC. It was hypothesized that PEG grafting would enhance solubility, reduce cytotoxicity, improve colloidal stability and maintain polyplex sizes under different conditions. Cellular binding and uptake of the polyplexes were confirmed using fluorescent-labeled DNA and evaluated using flow cytometry. The PEG-grafted TMC exhibited significantly higher uptake efficiency than the TMC, followed by the unmodified chitosan. The transfection efficiencies of TMC and PEG-grafted TMC were further evaluated in murine and human cell lines. At N/P = 30, PEG-grafted TMC enhanced transfection efficiency in three out of four cell lines as compared to TMC and unmodified chitosan (Fig. (3)). The transfection efficiency of PEG-grafted TMC-DNA complex was 4-fold (NIH/3T3 cells), 7-fold (MeWo cells) and 10-fold (L929 cells) higher than that of TMC-DNA complex (Fig. (3)). In MeWo cells, the transfection efficiency of PEG-grafted TMC-DNA and PEI-DNA complexes were comparable (Fig. (3)).

2.1.2. 6-Amino-6-Deoxy-Chitosan—6-amino-6-deoxy-chitosan (6ACT) can be prepared by deoxyhalogenation of N-phthaloylchitosan to introduce halogens at the carbon-6 positions of chitosan, followed by azidation at the carbon-6 positions, and then reduction of the azido groups [70, 71]. Satoh et al. [71] investigated the 6ACT as a gene carrier and evaluated the transfection efficiency in COS-1 cells. It was found that 6ACT and chitosan had similar cytotoxicity profiles, and both were generally less toxic than PEI. In the presence of serum, the optimal transfection efficiency for 6ACT-DNA complex (N/P = 2.5) was 2.5-fold higher than that for chitosan-DNA complex (N/P = 5). However, the transfection efficiency for 6ACT-DNA complex (N/P = 2.5) was only about a quarter of that for PEI-DNA complex (N/P = 10).

2.2. Hydrophobic Modification

Since chitosan is a hydrophilic polysaccharide, chitosan-based polymeric amphiphiles may be prepared by chemically attaching hydrophobic functional groups to chitosan. In general, such self-assembled colloidal systems are promising carriers of drugs, proteins, antigens and genes owing to their low cytotoxicity and enhanced therapeutic effect [72–76]. Indeed, they have captured significant attention mostly due to their useful properties [72, 73], which include but are not limited to: i) increased stability and half-life in the blood circulation, allowing for accumulation at pathological sites, ii) targeting capability owing to the various targeting ligands attached to the carrier systems, iii) enhanced intracellular penetration with the aid of surface-attached cell-penetrating molecules, iv) contrast properties owing to the loading of contrast agents for various imaging applications, and v) responsiveness to local biological stimuli such as pH and temperature, allowing the release of encapsulated agents.

Self-assembled polymeric amphiphiles are considered as one of the colloidal carrier systems and they are usually formed from either block co-polymers [77, 78] or hydrophobically modified water-soluble polymers [79, 80]. These polymeric amphiphiles can form self-assembled nanoparticles in an aqueous medium via hydrophobic interactions among the hydrophobic chains within the core, while exposing the hydrophilic chains outside the shell, primarily to minimize interfacial free energy. For chitosan, numerous hydrophobic functional groups have been used to develop chitosan-based amphiphiles such as alkyl

groups [81–85], fatty acids (e.g. stearic acid, linoleic acid, palmitic acid, octanic acid and oleic acid) [86–93] and bile acids (e.g. 5 β -cholanic acid, deoxycholic acid and cholic acid) [94–103]. By varying the degree of substitution of the hydrophobic functional groups, it is easy to control the required particle size and zeta potential of the nanoparticles. To date, hydrophobically modified chitosan amphiphiles have been widely used as a drug [81–83, 94, 95, 97, 98, 86–88, 90–92] and gene [84, 85, 101–103, 93] carrier to treat various hereditary or acquired diseases.

The introduction of hydrophobic functional groups to chitosan is expected to enhance transfection efficiency significantly. Kurisawa et al. [104] synthesized new copolymers with different compositions of N-isopropylacrylamide (IPAAm), 2-(dimethylamino)ethyl methacrylate (DMAEMA) and butylmethacrylate (BMA), where BMA was the hydrophobic component. The authors found that the transfection efficiency of the polymer-DNA complex in COS-1 cells was enhanced with increasing content of BMA in the polymer. The results were also consistent with findings by other researchers [105, 106]. It appeared that the introduction of hydrophobic groups contributed to increased transfection efficiency by modulating complex action with or within cells, such as surface adsorption and subsequent cellular uptake. In addition, hydrophobic groups in the polymeric carriers may aid in the dissociation of the polymer-DNA complex and subsequent release of DNA, which otherwise would be strongly bound through ionic interactions between the cationic carrier and the phosphate groups of DNA. These favorable features may result in higher transfection efficiency for hydrophobically modified chitosan than polymeric carriers using only ionic interactions.

2.2.1. N-Alkyl Functional Group—The incorporation of alkyl groups to chitosan to form N-alkylated chitosan (ACS) may be prepared by modifying chitosan with alkyl bromide [79, 84, 85]. It is found that chitosan which is substituted with alkyl groups having a minimum of six carbon atoms is likely to exhibit hydrophobic interactions [107]. Liu et al. [85] investigated the mechanism of interaction between the ACS and the cell membrane mimic, dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and explored the influence of alkyl side chains on the transfection efficiency in C₂C₁₂ cells. They found that ACS-DNA complex with a lower N/P had the same inhibition effect against DNase as compared to chitosan-DNA complex with a higher N/P. They considered that a small amount of ACS could play the same shielding role as chitosan. The interactions of naked DNA, chitosan-DNA and ACS-DNA complexes with DPPC membrane formed on mica substrates were evaluated. For naked DNA, they tended to cluster on the surface of DPPC, with no membrane perturbation. In contrast, significant membrane perturbation was observed for both chitosan-DNA and ACS-DNA complexes. From the transfection studies, the transfection level increased with the length of the alkyl groups, after which it reached a plateau. The transfection efficiency of chitosan-DNA complex was 4-fold higher than that of naked DNA (control group). For 4-CS-DNA (chitosan with 4-carbon alkyl group) complex, the efficiency was 5-fold higher than that of control group. For 8-CS-DNA, 12-CS-DNA and 16-CS-DNA (chitosan with 8-, 12-, and 16-carbon alkyl groups respectively) complexes, the efficiencies were about 7-fold higher than that of control group. It was possible that the higher transfection efficiency of ACS was attributed to the increasing entry into cells facilitated by hydrophobic interactions and easier unpacking of DNA from ACS carriers due to the weakening of electrostatic attractions between DNA and ACS.

Fatty acids can also be coupled to chitosan as a hydrophobic group via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) mediated coupling reaction [93]. Stearic acid, which is an endogenous long-chain saturated fatty acid, is widely accepted and used for pharmaceutical applications [110, 111]. Stearic acid is expected to be biocompatible with low cytotoxicity since it is a major component of fat. Hu et al. [93] verified that stearic acid

grafted chitosan oligosaccharide (SA-CSO) could efficiently compact DNA and protect them from nuclease degradation. From the time series transfection study, the gene expression mediated by Lipofectamine increased within the first 24 h before decreasing rapidly with time, possibly due to the high toxicity of the reagent. In contrast, the gene expression mediated by SA-CSO increased with time and was slightly lower than that of Lipofectamine.

2.2.2. Bile Acid Functional Group—Glycol chitosan (GC) can be hydrophobically modified with 5 β -cholanic acid in the presence of EDC and N-hydroxysuccinimide (NHS) to form a self-assembling polymeric amphiphile for gene delivery [80, 99, 100]. In order to efficiently encapsulate water-soluble DNA in the hydrophobically modified glycol chitosan (HGC), DNA is first hydrophobized with cetyltrimethylammonium bromide (CTAB) to favor the hydrophobic interactions within the core of the nanoparticles [80]. CTAB and DNA can rapidly self-aggregate into insoluble complexes in an aqueous phase with different charge ratios [108, 109]. Yoo et al. [80] had performed characterization studies on the HGC nanoparticles as well as evaluated their transfection efficiencies both in vitro and in vivo. High encapsulation efficiency, ranging between 88 and 97%, can be obtained. The uptake of the mixture of HGC/GC nanoparticles by COS-1 cells was confirmed by confocal microscopy, and nanoparticles with a higher HGC content were taken up by the cells more effectively due to the smaller particle sizes. The results obtained from the in vitro study were consistent with those from the in vivo study; a large amount of HGC content in the mixture of HGC/GC-DNA nanoparticles enhanced the transfection efficiency. For the in vitro study, HGC/GC-DNA nanoparticles with 100% HGC exhibited about 8-fold higher transfection efficiency than chitosan-DNA nanoparticles, and comparable efficiency with PEI-DNA nanoparticles. For the in vivo study, the transfection efficiency of HGC/GC-DNA nanoparticles with HGC content above 80% exceeded that of naked DNA and commercial transfection agent. In another study, Park et al. [99] investigated GC with different molecular weights, mainly GC-20 kDa-NP, GC-100 kDa-NP and GC-250 kDa-NP, that were modified with cholanic acid at the same molar ratio. Interestingly, the physicochemical properties of the nanoparticles, including the degree of substitution with cholanic acid, surface charge, particle size and in vitro stability, were similar regardless of the molecular weight. However, the tissue distribution and tumor accumulation behavior of GC nanoparticles were dependent on the molecular weight. In particular, hydrophobically modified GC-250 kDa-NP exhibited longer circulation time in the blood stream and were less likely to be excreted by the kidneys. Moreover, high amount of hydrophobically modified GC-250 kDa-NP accumulated at the tumor site for up to 3 days. The cellular uptake mechanism of the HGC nanoparticles was later tracked in the HeLa cells via the use of several endocytic inhibitors [100]. Uptake was reduced by 20% when chlorpromazine (inhibitor for clathrin-mediated endocytosis) was used. The uptake was reduced by 40% when filipin III (inhibitor for caveolae formation) was used. Interestingly, an additive inhibitory effect was shown when both chlorpromazine and filipin III were used at the same time. The uptake was reduced by 30% when amiloride (inhibitor for Na⁺/H⁺ exchange required for macropinocytosis) was used. In summary, the internalization of the nanoparticles was mediated by more than one cellular uptake mechanism. The colocalization of the HGC nanoparticles with lysosomal vesicles in cells was also confirmed based on the microscopy images.

Deoxycholic acid can be coupled to chitosan as an alternative hydrophobic group via an EDC-mediated coupling reaction [101, 102]. Kim et al. [102] prepared deoxycholic acid-modified chitosan (DAMC) of varying molecular weights (5–200 kDa) with a constant 6% degree of substitution in order to investigate their complexation behavior with DNA and their transfection efficiencies in COS-1 cells. The electrophoretic mobility of DNA decreased with increasing molecular weight of DAMC at the same N/P. Low molecular

weight DAMC could not completely retard DNA mobility, even at high N/P. In the absence of serum, DAMC40k-DNA (derivative from 40 kDa chitosan) complex exhibited the highest transfection efficiency. DAMC5k-DNA (derivative from 5 kDa chitosan) complex showed the lowest transfection efficiency, possibly due to its low stability at physiological ionic strength. DAMC200k-DNA (derivative from 200 kDa chitosan) complex showed lower transfection efficiency than DAMC40k-DNA complex, possibly due to its rather strong interaction with DNA. In the presence of serum, the transfection efficiencies of various DAMC were much lower. In particular, the transfection efficiency of DAMC40k-DNA complex was reduced by about 200 times. In another study, Chae et al. [103] prepared DAMC from two molecular weights of chitosan, mainly 1~3 and 3~5 kDa, with 5 and 10% degrees of substitution. Similarly, the authors observed a molecular weight dependent electrophoretic mobility of DNA. In HEK 293 cells in the absence of serum, the most efficient carrier COS3D25 (1~3 kDa chitosan with 5% degree of substitution) mediated more than 20- and 30-fold enhancement of transfection efficiency compared to poly(L-lysine) (PLL) and COS3 (1~3 kDa chitosan), respectively (Fig. (4a)). In addition, the transfection efficiency decreased with increasing degree of substitution (Fig. (4a)). It is interesting that, in the presence of serum, the transfection efficiencies of the DAMC were not significantly affected, but PLL transfection was (Fig. (4b)). In comparison with the transfection efficiency in the absence of serum, 88% relative transfection efficiency was obtained in serum by COS3D25 (Fig. (4b)). The transfection efficiency of COS3D25-DNA complex was 100-fold higher than that of PLL-DNA complex (Fig. (4b)). Thus, the complexation behavior of the carriers with DNA and their transfection efficiencies are highly dependent on the molecular weight and the degree of substitution of hydrophobic groups.

2.3. Targeting Ligands

2.3.1. Galactose Ligand—Galactose ligand may be introduced to chitosan for liver-targeted delivery. It is well established that the asialoglycoprotein receptor (ASGP-R) is present at high density only on hepatocytes and retained on several human hepatoma cell lines [112–116]. These receptors recognize glycoproteins having terminal galactose or glucose residues on their oligosaccharide chains. Though there have been numerous methods for liver-targeted gene delivery mediated by non-viral gene carriers [117–119], gene delivery by ASGP-R is known to be one of the most effective approaches as demonstrated by promising in vivo results [120, 121].

Galactosylated chitosan (Gal-C) has been shown to be a safe and effective carrier that is able to condense DNA and deliver them to the targeted cells. The Gal-C-DNA complexes are stable and are able to stay in the plasma circulation for prolonged periods of time. This is especially important for intravenous injection in which a long circulation time is needed to allow the complexes to reach the targeted cells. Kim et al. [122] conducted a time series of turbidity measurements on Gal-C-DNA complexes, in which an increase in turbidity indicated self-aggregation while a decrease indicated precipitation. It was found that for N/P ranging from 1 to 5, the turbidity decreased rapidly with time. For N/P from 10 to 20, the turbidity showed gradual increase for 3 days, followed by a drastic decrease. In addition, when the complexes were incubated in serum, they were still maintained after 7 days. The cytotoxicity of Gal-C was further evaluated in HepG2 and HeLa cells, with comparison to PEI. In particular, Gal-C had no effect on cell viability in both cell lines and average cell viability over 100% was obtained. However, for PEI, low cell viability of about 20 to 40% at concentration ranging from 5 to 25 $\mu\text{g/ml}$ was obtained. In another study, Gao et al. [123] evaluated the suitability of galactosylated low molecular weight chitosan (Gal-LMWC) as a vector for antisense oligonucleotide and plasmid DNA for hepatocyte-targeted delivery. The authors found that high molecular weight chitosan (HMWC), low molecular weight chitosan

(LMWC) and Gal-LMWC were less cytotoxic than pure transfection agents, including calcium phosphate and Lipofectin. LMWC was less cytotoxic than HMWC. On the contrary, Gal-LMWC exhibited no cytotoxicity at the same concentration. It appeared that modification reduced the cytotoxicity of HMWC due to the decrease of the positive charge density.

A number of synthetic approaches for other galactosylated compounds have also been widely reported in the literature [124–127]. Park et al. [124] prepared and evaluated galactosylated chitosan-graft-dextran (Gal-C-D) as a potential hepatocyte-targeting gene carrier. Dextran was grafted to Gal-C for enhancing stability of the complex in aqueous media. The Gal-C-D-DNA complexes were found to efficiently transfect Chang liver cells and HepG2, which express ASGP-R. In parallel work, Park et al. developed galactosylated chitosan-graft-poly(ethylene glycol) (Gal-C-PEG) [125] and galactosylated chitosan-graft-poly(vinyl pyrrolidone) (Gal-C-PVP) [126] for the same purpose. Gal-C-PEG-DNA complexes were found to be relatively stable due to the presence of hydrophilic PEG shielding and enhanced protection against nuclease degradation. In a similar way, Gal-C-PEG-DNA complexes were found to transfect HepG2 cells efficiently. Besides, Gal-C-PVP exhibited improved physicochemical properties. In another study, Lin and Chen [127] synthesized chitosan-O-PEG-galactose by substituting the hydroxyl groups of chitosan with PEG polymer chains. The PEGylated chitosan possessed amphiphilic property and was soluble in both neutral aqueous media and organic solvents. At the same time, it also possessed sufficient quantity of targeting moieties for ASGP-R on hepatocytes.

2.3.2. Mannose Ligand—The mannose receptor is a critical pattern recognition receptor of innate immunity and may be found on the surface of macrophages and dendritic cells, including alveolar macrophages [128], murine resident peritoneal macrophages, thioglycollate-elicited peritoneal macrophages, macrophages derived from bone marrow [129], human monocyte-derived macrophages [130], and immature dendritic cells [131]. Thus, the mannose receptor is a key effector molecule in inducing functions of these cells and in initiating an early immune response against invading pathogens. The modulation of this receptor expression is important in the role of these cells in antigen processing, scavenging, and host defense against pathogens. Due to the central role of macrophages and dendritic cells in the initiation of primary immune responses, there is high interest in employing mannose-conjugated carriers for targeted delivery of drug and gene for immunotherapy of diseases such as cancer.

Hashimoto et al. [132] conjugated chitosan with mannose of two different degrees of substitution, mainly 5 and 21%, to form mannosylated chitosan (Man-C) and evaluated their transfection efficiencies in mouse peritoneal macrophages and COS7 cells. Transfection of mouse peritoneal macrophages by both 5% Man-C-DNA and 21% Man-C-DNA complexes was significantly higher than that of unmodified chitosan-DNA complex, and was comparable to that of mannosylated PEI-DNA complex. For COS7 cells, the transfection efficiency of 5% Man-C-DNA complex was comparable to that of unmodified chitosan-DNA and Lipofectin-DNA complexes, and was higher than that of 21% Man-C-DNA complex. Confocal fluorescence micrographs also confirmed the cellular uptake of Man-C-DNA complexes into macrophages. In contrast, unmodified chitosan-DNA complexes were located mainly near the cell surface. The authors suggested that the balance between DNA condensation and receptor recognition was critical in optimizing the transfection efficiency and indicated that a degree of substitution of 5% was sufficient. In another study, Kim et al. [133] investigated the effects of chitosan-DNA and Man-C-DNA (7.3% degree of substitution) nanoparticles containing the murine interleukin-12 (mIL-12) gene to enhance anti-tumor immune response in dendritic cells of the tumor. The *in vitro* efficiencies of the nanoparticles in improving cytokine production were monitored in dendritic cells collected

from mice. In particular, the cytokine levels of mIL-12 p70 and mIFN- γ were found to be significantly higher for both nanoparticle formulations than those for naked mIL-12 plasmid. Moreover, the Man-C-pmIL-12 nanoparticles mediated higher cytokine production than the unmodified chitosan-pmIL-12 nanoparticles. Consistent results were also obtained for the in vivo efficiencies of the nanoparticles evaluated in BALB/c mice bearing CT-26 carcinoma cells. Man-C-pmIL-12 nanoparticles mediated higher cytokine levels of mIL-12 and mIFN- γ than the control and the vector control. In addition, the administration of Man-C-pmIL-12 nanoparticles significantly suppressed tumor growth and angiogenesis and effectively induced cell cycle arrest and apoptosis.

2.3.3. Folate Ligand—Folate is a high affinity ligand that can enhance the differential specificity of conjugated carriers for drug and gene by targeting folate receptors of cancerous cells. Folate receptors are over-expressed in many carcinomas, including ovary, kidney, lung, mammary gland, brain, endometrium and myeloid cancerous cells, while they are found at low levels in most normal tissues [134, 135]. The folate receptor, a tumor associated glycosyl phosphatidylinositol-anchored protein, can actively internalize bound folate and folate-conjugated compounds via receptor-mediated endocytosis [136].

Zheng et al. [137] studied the cellular uptake and transfection efficiency of folate conjugated N-trimethyl chitosan (Fol-TMC) on both folate receptor over-expressing cells (KB and SKOV3 cells) and folate receptor-deficient cells (A549 and NIH/3T3 cells) in vitro, and compared to those of the unconjugated TMC. The cellular uptake of Fol-TMC-DNA complex, determined by flow cytometry, was significantly higher than that of TMC-DNA complex. Though the uptake of Fol-TMC-DNA complex could be accomplished by more than one pathway (both folate receptor-mediated and receptor-independent endocytosis), the transfection efficiency of the Fol-TMC-DNA complex was only 1.6- and 1.4-fold higher than that of the TMC-DNA complex in KB and SKOV3 cells, respectively. The authors suggested the following reasons for the observation: i) the existence of receptor-independent endocytosis made the impact of folate receptor-mediated endocytosis less important, ii) the folate was directly conjugated to TMC without any flexible polymer chain that could have improved the spatial freedom of the ligand and favored the interaction between the ligand and the receptor, and iii) the rate of folate receptor-mediated endocytosis as compared to that of non-specific endocytosis was faster than expected, and the results were obtained far after the complex reached the cell nucleus.

The expression of folate receptor is also found in synovial mononuclear cells and CD14⁺ cells from patients with rheumatoid arthritis [138]. In fact, articular macrophages isolated from rats with adjuvant-induced arthritis over-express folate receptors and show significantly higher binding capacity for folate conjugates than macrophages obtained from healthy rats [139]. Thus, folate also serves as a potential ligand for carriers in the treatment of rheumatoid arthritis. Fernandes et al. [140] investigated folate conjugated chitosan (Fol-C) nanoparticles containing the interleukin-1 receptor antagonist (IL-1Ra) gene on controlling bone metabolism and inflammation in a rat adjuvant-induced arthritis model of rheumatoid arthritis via the usage of inflammation and bone turnover markers. The delivery of IL-1Ra gene significantly reverted the alterations in bone turnover by modulating the level of osteocalcin as well as the activities of alkaline phosphatase and tartrate-resistant acid phosphatase. The protective effects of the nanoparticles were also reflected by the decrease in expression of interleukin-1 β and prostaglandin E₂ as well as osteoclast number and other histopathological findings. In comparison with chitosan-IL-1Ra nanoparticles and naked IL-1Ra plasmid, it was found that Fol-C-IL-1Ra nanoparticles provided better protection against inflammation and bone damage.

3. POLY(AMIDOAMINE) DENDRIMERS

PAMAM dendrimers are a relatively new class of polymers that are highly branched with well-defined and three-dimensional structures. They consist of tertiary amines in the core, primary amines on the surface and an amide backbone as shown in Fig. (5). In general, dendrimers can be synthesized by two methods: the divergent and convergent methods. The divergent synthesis approach, pioneered by Tomalia [141], involves outward branching from an initiator core by a series of stepwise polymerization reactions that attach layers to form dendrimers of various generations. The convergent synthesis approach, introduced by Hawker and Frechet [142], involves inward branching from what will become the dendrimer surface to the inner core by the formation of individual dendrons. The dendron is produced by a reiterative coupling of branch units, and the process is followed by a core anchoring step, enabling the formation of dendrimers of various generations. Though both synthesis approaches pose some challenges towards large-scale production of dendrimers [143], alternative methods have been proposed [144–147]. The choice of the initiator core, the branching cells and the external surface functional groups can bring about unlimited types of dendrimers. PAMAM dendrimers are synthesized by the divergent approach, and an initiator core based on ethylene diamine or ammonia is usually used. With the addition of a new layer, the molecular weight of the dendrimer increases exponentially, the number of primary amine groups on the dendrimer surface doubles, and the diameter increases by about 10 Å [148]. In addition, PAMAM dendrimers have a low polydispersity. As the dendrimer generation increases, the density of the surface branching units begins to impart various structural effects on the dendrimer shape. In particular, dendrimers of lower generations have a planar and elliptical shape whereas dendrimers of higher generations have a spherical conformation [148]. For the latter case, the interior core of the dendrimer develops a hydrophobic space which is useful for the encapsulation of compounds for various pharmaceutical applications [148].

PAMAM dendrimers have attracted the interest of many researchers over the past few years. In comparison with other types of delivery systems, the advantages of using dendrimers include: i) controllable structure and size, ii) high chemical and structural homogeneity, and iii) high ligand and functionality density, all of which can be optimized for specific delivery purpose. While PAMAM dendrimers do not induce significant cytotoxicity in vitro [149, 150] or in vivo [151, 152], the biocompatibility of these polymers is dependent on the size, surface charge and concentration [153–157]. PAMAM dendrimers are also found to be able to mediate transport through the epithelial barrier, indicating their potential as carriers for oral delivery [157–160]. Besides, PAMAM dendrimers are also known to be efficient gene delivery systems. They have been shown to be as efficient as or more efficient than other cationic carriers such as polylysine for gene transfer [151]. PAMAM dendrimers have primary and tertiary amine groups, which differ in their pKa values. The surface primary amine groups have a pKa value in the range of 7 to 9, whereas the interior tertiary amine groups have a pKa value in the range of 3 to 6 [161]. The surface primary amine groups participate in DNA binding and promote cellular uptake of these complexes. On the other hand, the interior tertiary amine groups exert endosome buffering effect and enhance the release of DNA in the cytoplasm.

PAMAM dendrimers allow the precise control of size, shape and attachment of functional groups that are desirable for various pharmaceutical applications. In particular, they are highly efficient carriers for gene delivery. Over the years, we have seen a great deal of research efforts which are aimed at enhancing PAMAM dendrimers for better cell targetability and optimizing localized transgene expression. The details will be discussed in the following sections. Selected examples of PAMAM dendrimer derivatives are shown in Fig. (6).

3.1. Generations of Poly(amidoamine) Dendrimers

The cytotoxicity of PAMAM dendrimers is dependent on concentration and generation of the dendrimer. Roberts et al. [152] investigated the cytotoxicity of PAMAM dendrimers (G3, G5 and G7) both in vitro and in vivo. For cytotoxicity tests in vitro on V79 cells, PAMAM G3 affected cell growth at 1 mM whereas PAMAM G5 showed cytotoxicity at 10 μ M. In both cases, the cell viability was less than 10%. Cell growth was greatly affected by PAMAM G7, in which less than 10% cell viability was obtained even at a low concentration of 100 nM. For cytotoxicity tests in vivo, no significant cytotoxicity or immune response was detected. A series of biodistribution studies of the PAMAM dendrimers was also conducted. Interestingly, the pancreas exhibited remarkable accumulation of the dendrimers, and high urinary output, particularly for PAMAM G7, was observed.

The dendrimer generation needed to achieve optimal transfection efficiency can be determined. However, the specific dendrimer most efficient in achieving transfection varied among different types of cells. Kukowska-Latallo et al. [149] reported that PAMAM dendrimers were highly efficient in transfecting a wide variety of cells with minimal cytotoxicity. However, the ability to transfect cells sometimes required additional agents such as diethylaminoethyl dextran, which appeared to alter the nature of the complex, or chloroquine, which acted as a lysosomotropic agent. The optimal generation of PAMAM dendrimers based on transfection assays performed on different cell lines was determined. Most of the cell lines could attain optimal gene expression with dendrimer generation ranging from G7 to G10. Haensler and Szoka [162] investigated a series of PAMAM dendrimers (G2 to G10) and reported that the transfection efficiency in CV-1 cells was a function of both N/P and diameter of dendrimer. In particular, high luciferase expression required an excess of dendrimer to DNA. In addition, the large diameter dendrimers (beyond G4, 40 nm) mediated better transfection efficiencies than the small ones. The complex formed by PAMAM G6 at N/P = 6 showed about 1000-fold higher transfection efficiency than that formed by PLL, and about 100-fold higher than that formed by cationic lipid DOTMA. The authors explained that an excess of dendrimer to DNA was required for effective adsorptive endocytosis and membrane destabilization. Moreover, the enhancement in luciferase expression from PAMAM G4 onwards may be attributed to a structural change in the polymer; PAMAM G3 resembled a starfish whereas PAMAM G5 had a spherical form. Thus, it appeared that the spherical shape aided in condensing the DNA in the highly structured core and destabilizing the cell membrane.

Bielinska et al. [150] evaluated a series of PAMAM dendrimers (G6, G7 and G10) for the delivery of antisense oligonucleotides. The authors investigated the inhibition of luciferase expression from pCMV-Luc plasmid cotransfected with antisense-ATG oligonucleotides on D5 cells at different N/P. All the dendrimer-antisense-ATG oligonucleotides were effective in inhibiting luciferase expression. In comparison with the plasmid alone transfected without oligonucleotides or plasmid transfected with sense-ATG oligonucleotides, the plasmid transfected with antisense-ATG oligonucleotides showed the maximum decrease of about 50% in luciferase expression with PAMAM G7 at N/P = 10. Moreover, the retention and stability of the antisense-ATG oligonucleotides, either alone or complexed with a series of dendrimers (G5, G7 and G9), were also tested on U937, D5, Rat2 and Jurkat cells at various N/P. Dendrimers enhanced the uptake of oligonucleotides in U937 cells by about 4- to 5-fold as compared to the free oligonucleotides and provided longer retention times up to 4 days, with no significant difference among the generations of dendrimers. Similar results were observed for the other three cell lines.

The generation number affects the microvascular extravasation of PAMAM dendrimers. Tajarobi et al. [163] studied the transepithelial transport of a series of PAMAM dendrimers (G0 to G4) across Madin-Darby canine kidney cells in vitro. It was found that the

permeability of the dendrimer was the highest for PAMAM G4, followed by G1 or G0, G3 and G2, and was governed by a balance between the size of the polymer and its interaction with the cells. Results suggested that the cell integrity was compromised in the presence of PAMAM G4, thus leading to high permeability. In another study, Zhang and Smith [164] focused on higher generations of PAMAM dendrimers (G4 to G7) and studied their membrane disruption properties. Similarly, the authors showed that higher generation dendrimers, especially for PAMAM G6 and G7, were effective in disrupting or influencing the integrity of the cell membranes as determined by standard fluorescence assays for lipid mixing, leakage and contents mixing. El-Sayed et al. [165] also investigated the factors influencing the microvascular extravasation of PAMAM dendrimers (G0 to G4) across muscle tissues of hamsters in vivo and compared the dendrimers with a linear PEG of similar molecular weight. The authors reported extravasation of the fluorescent-labeled dendrimers in terms of extravasation time, a parameter measuring the rate of extravasation or transvascular transport across microvascular endothelium into the interstitial tissue. The extravasation of the dendrimers clearly showed size and molecular weight dependence. In particular, the increase in both size and molecular weight of the dendrimers led to exponential increase in the extravasation time from 143.9 to 422.7 s for PAMAM G0 to G4. It appeared that as the molecular diameter of the dendrimer increased, the exerted viscous drag on it together with its degree of exclusion from the endothelial pores increased, thus explaining the increase in extravasation time. Interestingly, the extravasation time of PEG (6000 Da) did not follow the expected molecular weight trend exhibited by the dendrimers. Instead, the extravasation time of PEG was longer than that of PAMAM G4 (14,215 Da). The authors suggested that the hydrated PEG chains followed a coiled conformation and conferred them a larger hydrodynamic volume, resulting in a longer extravasation time.

3.2. Activated and Non-Activated Poly(amidoamine) Dendrimers

Activation of PAMAM dendrimers involves hydrolytic cleavage of some of the amide bonds in the inner part of the polymer and removal of some of the dendrimer branches. The process involves significant degradation of the dendrimer, resulting in a mixed population of compounds with molecular weights ranging from the very low (<1500 Da) to several tens of kilodaltons [166]. Carboxyl groups are formed at the cleavage sites and the resulting dendrimer has a higher degree of flexibility. The overall size and shape of the dendrimer, however, remained the same following the activation process. Hydrolytic cleavage of the amide bonds can be performed by solubilizing the dendrimer in an appropriate solvolytic solvent and heating for a certain period of time. It has been suggested that heat activation of the dendrimers can be performed in any solvolytic solvent, although the heating time required to achieve maximum activity will be dependent on the solvent used [166].

Navarro and Tros de ILarduya [167] characterized dendriplexes prepared with PAMAM G4 and G5, and compared the differences between activated and non-activated forms. In terms of particle sizes, for non-activated dendriplexes, there were no notable differences by increasing N/P in G4 or G5 dendriplexes. However, for activated dendriplexes, the sizes decreased slightly in both G4 and G5 dendriplexes with increasing N/P. The zeta potentials of both non-activated and activated dendriplexes generally increased with increasing N/P, especially for G5 dendriplexes. Overall, there were no notable differences in the surface charge of dendriplexes by adjusting the activation time or by using a different dendrimer generation.

Activated PAMAM dendrimers mediate higher transfection efficiency as compared to non-activated PAMAM. Tang et al. [166] evaluated the transfection efficiencies of activated (or fractured, as described) PAMAM dendrimers on CV-1 cells. In the study involving PAMAM G6 and G8 based on ethylene diamine core (6EDA and 8EDA, respectively) as well as PAMAM G5 and G6 based on tris(2-aminoethyl)amine core (5TAEA and 6TAEA,

respectively) dendrimers, it appeared that there was an optimal heat treatment time for the maximum transfection efficiency to be achieved. The lower generation dendrimers needed less heat treatment time to achieve maximum transfection efficiency, although their corresponding efficiencies were lower than those of higher generation dendrimers. For the case of activated 6TAEA dendriplex, it enhanced transfection efficiencies by about 100- and 1000-fold as compared to that of intact dendriplex for β -galactosidase and luciferase assays, respectively. The authors suggested that the flexibility of the activated dendrimers played a critical role in the transfection mechanism. Navarro and Tros de ILarduya [167] also compared the transfection efficiencies of activated and non-activated PAMAM dendrimers both in vitro and in vivo. The authors observed a similar trend in the effect of heat treatment time of the dendrimers on the transfection efficiency of activated dendriplex in HepG2 cells. The authors explained that if the activation time was too short, the dendrimer would not be flexible enough for efficient transfection. On the other hand, if the activation time was too long, the dendrimer would not have sufficient charge density necessary for complex formation and/or would not have sufficient mass to swell and rupture the endosome. The maximum transfection efficiency observed for activated dendriplex based on PAMAM G4 was about 4-fold ($N/P = 4$ and 6) higher than that for the corresponding non-activated dendriplex. For activated dendriplex based on PAMAM G5, the efficiency was about 2-fold ($N/P = 4$) and 7-fold ($N/P = 6$) higher than that for the corresponding non-activated dendriplex. A similar trend was also observed for in vivo studies on mice, especially for the transfection efficiency obtained in the lungs. These results indicate that both generation number and heat treatment time can be optimized to enhance activated PAMAM dendrimer as a gene carrier.

3.3. Quaternized Poly(amidoamine) Dendrimers

Recently, quaternary ammonium salt has been introduced to the internal tertiary amine of surface-acetylated PAMAM dendrimer (PAMAM-NHAc) and hydroxyl-terminated PAMAM dendrimer (PAMAM-OH) by methylation to improve gene delivery. These quaternized PAMAM dendrimers possess several advantages including: i) the modified neutral surface of the dendrimer offers lower cytotoxicity and overcomes aggregation problem, ii) the presence of cationic charges located in the core of the dendrimer provides binding sites for negatively charged DNA, iii) beyond a certain degree of quaternization, the polyplexes of quaternary amine-based polymers are relatively smaller and more compact compared to those prepared by cationic polymers containing primary and tertiary amine groups, indicating that the particles are small enough to be taken up by receptor-mediated endocytosis, and iv) quaternary amine-based polymers are cationic at most pH values as a strong electrolyte, while the charge density in the primary amine-based polymers depend on the pH of the media. Besides gene delivery [168–171], quaternary ammonium groups are introduced to dendrimers for a number of other applications [172–175].

Quaternization is useful for the reduction of cytotoxicity of unmodified PAMAM dendrimers. Lee et al. [168] observed a positive correlation between the cell viability of HEK 293T cells and the degree of quaternization of the ammonium salts synthesized based on PAMAM G4. With the degree of quaternization ranging from 10 to 70%, the minimum cell viability exposed to quaternized PAMAM dendrimers at concentrations up to 100 $\mu\text{g/ml}$ was found to be about 75% relative to the untreated cells and showed low cytotoxicity as compared to unmodified dendrimers and PEI. In another study, Lee et al. [169] found a similar trend for the case of quaternized PAMAM-OH dendrimers. In this case, the relative cell viability for quaternized dendrimers was over 90% at all concentrations tested (up to 100 $\mu\text{g/ml}$). The higher cell viability may be attributed to the shielding of the interior positive charges by surface hydroxyl groups.

Lee et al. [168] showed that the degree of quaternization in PAMAM dendrimers influenced the transfection efficiency. For transfection studies on HEK 293T cells, the levels of luciferase expression mediated by 0.5-QPAMAM and 0.7-QPAMAM (50 and 70% degree of quaternization respectively) were lower than that of 0.1-QPAMAM (10% degree of quaternization). The expression level of 0.1-QPAMAM was similar to that of unmodified dendrimer. The expression levels mediated by 0.5-QPAMAM and 0.7-QPAMAM exhibited a 10-fold decrease as compared to that of unmodified dendrimer. It seemed that the quaternized PAMAM dendrimers lacked responsiveness on endosomal escape. In another study, Lee et al. [169] found a similar trend for the case of quaternized PAMAM-OH dendrimers. In this case, the transfection efficiencies of complexes formed by various quaternized PAMAM-OH dendrimers (52, 78 and 97% degrees of quaternization) were lower than those of complexes formed by unmodified dendrimer and PEI. It seemed that the neutral surface of PAMAM-OH dendrimers resulted in less interactions with the cell membrane, thus causing lower transfection efficiencies. Recently, Patil et al. [171] improved the delivery of siRNA by varying the degree of quaternization in PAMAM-OH dendrimers. Cellular internalization of siRNA in A2780 human ovarian cancer cells showed that dendrimers with a lower degree of quaternization (20 to 30%) delivered siRNA more efficiently than dendrimers with a higher degree of quaternization (70 to 85%). However, the complexes of siRNA with PAMAM-OH dendrimers of different degrees of quaternization (20, 70 and 97%) did not show significant differences in the suppression of gene expression level. The authors also reported that the internal tertiary amine groups of PAMAM dendrimers played a significant role in the proton sponge effect. In particular, they believed that the almost complete quaternization of the tertiary amine groups of PAMAM-OH dendrimer was the main reason for the low efficacy in siRNA delivery.

3.4. Targeting Ligands

3.4.1. Cyclodextrin Functional Group—Cyclodextrins (CyD) are cyclic oligomers of 6, 7 or 8 glucose units, called α -, β -, and γ -cyclodextrins. CyD are cup-shaped molecules that have a hydrophobic cavity and a hydrophilic exterior, for which these properties are desirable for pharmaceutical applications. Davis and coworkers [176] reported the first example of linear cationic polymers containing β -CyD in the polymer backbone for gene delivery. Since then, the group has synthesized a set of such CyD-containing polymers and studied their structural effects on gene delivery [177–179]. Polycations incorporated with CyD can mediate high water solubility, low toxicity and efficient gene transfection in cell cultures. Moreover, CyD can extract cell membrane components such as phospholipids and cholesterol, subsequently leading to hemolysis and a change in membrane permeability of these complexes [180–182].

The effect of PAMAM dendrimers conjugated with different types of CyD has been investigated. Arima et al. [183] synthesized a series of PAMAM G2 dendrimers conjugated with α -, β - and γ -CyD (CyD conjugates) and compared their transfection efficiencies to commercial transfection agents, Lipofectin and TransFast. From the transfection studies on NIH/3T3 and RAW264.7 cells, it was found that α -CyD conjugate mediated superior transfection efficiency as compared to β - and γ -CyD conjugates. α -CyD conjugate-DNA complex had enhanced the luciferase activity significantly as compared to dendrimer-DNA complex over a wide range of N/P. At high N/P, α -CyD conjugate mediated higher transfection efficiency than that of Lipofectin, but lower than that of TransFast. In an attempt to improve the efficacy, the effect of α -CyD conjugates with PAMAM dendrimers of different generations (G2, G3 and G4) on transfection efficiencies were investigated in a separate study [184]. For both NIH/3T3 and RAW264.7 cells, α -CyD conjugate based on PAMAM G3 achieved the highest transfection efficiency as compared to α -CyD conjugate based on PAMAM G2 and G4, and it was comparable to TransFast. In both studies, it is

interesting to note that despite the presence of CyD, CyD conjugates exhibited negligible cellular association, thus suggesting negligible perturbation of the plasma membrane barrier by CyD. However, CyD conjugates influenced the intracellular trafficking of DNA, possibly in terms of the increase in the release of DNA from the endosome into cytoplasm after endocytosis.

The membrane-disruptive ability, cytotoxicity and transfection efficiency of CyD conjugates are dependent on the degree of substitution of CyD in PAMAM dendrimers. Kihara et al. [185] investigated the effect of degree of substitution of α -CyD in CyD conjugates based on PAMAM G3 dendrimer, mainly with 1.1, 2.4 and 5.4 degrees of substitution, on the above-mentioned properties. Results indicated that only α -CyD conjugates with higher degrees of substitution (i.e. 2.4 and 5.4) showed membrane-disruptive properties. However, α -CyD conjugates with high degrees of substitution, especially the one with degree of substitution of 5.4, showed significant cytotoxicity at high N/P. The α -CyD conjugate with degree of substitution of 2.4 exhibited superior transfection efficiency in NIH/3T3 and HepG2 cells at high N/P, in comparison to other conjugates with degrees of substitution of 1.1 and 5.4; the highest gene expression of α -CyD conjugate-DNA complex with degree of substitution of 2.4 was attained at N/P = 400 in both cell lines. From the in vivo transfection studies on BALB/c mice, consistent results were obtained; α -CyD conjugate with degree of substitution of 2.4 showed higher transfection efficiency as compared to unmodified dendrimer and other α -CyD conjugates in spleen at 12 h as well as in liver and kidney at 24 h after administration. These results indicate that the degree of substitution of CyD can be adjusted to optimize the design of CyD conjugates for gene delivery.

Recently, Tsutsumi et al. [186] evaluated the effect of α -CyD conjugate on siRNA-mediated gene-silencing and compared with commercial transfection agents including Lipofectamine 2000, TransFast and Lipofectin. The inhibition ratio of the complex system was calculated and the coefficient of variation of luciferase activity was reported. It was found that the α -CyD conjugate system showed the highest inhibition ratio and the lowest CV value, suggesting the most prominent RNAi effect of the α -CyD conjugate system.

3.4.2. Arginine Functional Group—Conjugation of arginine residues to the surface of PAMAM dendrimer has been reported to enhance transfection efficiency significantly. Arginine is an abundantly observed amino acid in cell penetrating peptides such as the protein transduction domain or Tat sequence of HIV, which are capable of efficiently penetrating cell membranes as well as carrying various cargos into cells [187].

Choi et al. [188] designed an arginine-grafted-PAMAM dendrimer (PAMAM-Arg) consisting of about 58 out of 64 surface primary amine groups coupled with arginine residues on PAMAM G4 and demonstrated its potential as a novel gene carrier in HepG2 and Neuro 2A cells. In HepG2 cells, the transfection efficiency of PAMAM-Arg-DNA complex was more than 10-fold higher than that of unmodified dendrimer-DNA complex, and was comparable to that of PEI-DNA complex (Fig. (7a)). Similarly, in Neuro 2A cells, the transfection efficiency of PAMAM-Arg-DNA complex was more than 100-fold higher than that of Lipofectamine and unmodified dendrimer-DNA complex, and was more than 10-fold higher than that of PEI-DNA complex (Fig. (7b)). In another study, Kim et al. [189] investigated the structure-activity relationship of arginine conjugation in the form of mono- and di-arginine conjugated PAMAM G3 and G4 dendrimers. Mono-arginine conjugated PAMAM G3 (PAMAM3-R) and G4 (PAMAM4-R) dendrimers had 31 (out of 32 surface primary amine groups) and 60 (out of 64 surface primary amine groups) arginine residues attached respectively. Di-arginine conjugated PAMAM G3 (PAMAM3-R2) and G4 (PAMAM4-R2) dendrimers had 59 and 116 arginine residues attached respectively. Studies on HeLa, HEK 293 and A549 cells showed that complexes formed by di-arginine

conjugated dendrimers generally led to higher transfection efficiencies than mono-arginine conjugated dendrimers. In particular, PAMAM3-R2 mediated higher transfection efficiency than PAMAM3-R in the presence or absence of serum. However, PAMAM4-R2 mediated higher transfection efficiency than PAMAM4-R only in the presence of serum in HeLa and A549 cells. Intracellular trafficking of the complexes was confirmed by microscopy for which the images showed that all the complexes were internalized into the cytosol, but only complexes formed by PAMAM3-R2 and PAMAM4-R2 were found within the nuclei. Unfortunately, di-arginine conjugated dendrimers induced severe cytotoxicity in the cells that was comparable to PEI.

3.4.3. Glucocorticoid Functional Group—Studies on enhancing the transport of PAMAM dendriplexes into the cell nucleus via glucocorticoid conjugation have been made. There are two main advantages of glucocorticoid conjugation [190]: i) the glucocorticoid receptors are nuclear receptors that exist in the cytoplasm without ligands, thus providing nuclear targeting of therapeutic macromolecules, and ii) the glucocorticoid receptors induce nuclear pore complex dilation with sizes up to 60 nm, thus facilitating translocation of molecules from the cytoplasm to the nucleus.

Choi et al. [191] prepared PAMAM-dexamethasone (PAMAM-Dexa) (4 dexamethasone residues on PAMAM G4) and evaluated its transfection efficiency in HEK 293 and Neuro 2A cells. For HEK 293 cells, in the absence of serum, the transfection efficiency of PAMAM-Dexa-DNA complex was found to be 2-fold higher than that of unmodified dendrimer-DNA complex, but lower than that of PEI-DNA complex (Fig. (8a)). In the presence of serum, the transfection efficiency of PAMAM-Dexa-DNA complex was superior to that of unmodified dendrimer-DNA and PEI-DNA complexes (Fig. (8a)). For Neuro 2A cells, in the absence of serum, the transfection efficiency of PAMAM-Dexa-DNA complex was found to be more than 2-fold higher than that of PEI-DNA complex, and more than 6-fold higher than that of unmodified dendrimer-DNA complex (Fig. (8b)). In the presence of serum, the transfection efficiency of PAMAM-Dexa-DNA complex was about 10-fold higher than that of PEI-DNA and unmodified dendrimer-DNA complexes (Fig. (8b)). In addition, images taken from confocal microscopy confirmed the accumulation of more PAMAM-Dexa-DNA complex in the nucleus region than unmodified dendrimer-DNA complex.

In a similar study, Ma et al. [192] investigated a glucocorticoid of high potency, triamcinolone acetonide, conjugated to PAMAM dendrimer to form PAMAM-TA with different degrees of substitution, mainly PAMAM-TA-L (0.22 triamcinolone acetonide residues on PAMAM G4) and PAMAM-TA-H (1.65 triamcinolone acetonide residues on PAMAM G4). Similar to Choi's results, regardless of the presence of serum, PAMAM-TA enhanced transfection efficiency in both HEK 293 and HepG2 cells as compared to unmodified dendrimer, though there was no significant difference in expression between PAMAM-TA-L and PAMAM-TA-H at the respective optimal weight ratio. Images taken from confocal microscopy also confirmed that PAMAM-TA could be translocated from the cytoplasm into the cell nucleus efficiently.

3.4.4. Transferrin Functional Group—The capillary endothelial cells of the brain display various specific transport proteins, including glucose and amino transporters, which are important in mediating biological molecules across the blood-brain barrier into the brain [193–195]. Transferrin receptors are found on the luminal membrane of brain endothelial cells and they mediate the endocytosis of the iron-transport protein transferrin into the cells [196, 197]. It is thought that the brain obtains the required iron via the transport of iron-loaded transferrin through the blood-brain barrier. Thus, transferrin seems to be a promising brain-targeting ligand. Besides, transferrin receptors are expressed in large amounts on

proliferating cells and on cells which have undergone malignant transformation. High levels of transferrin receptors have been described in various malignant tissues such as human breast carcinoma and breast sarcoma [198], human leukemia cells [199] as well as other carcinoma cells of pancreas, melanoma, bladder, rectum, neuroblastoma, oat cells and teratoma [199]. Thus, transferrin can provide targeted delivery to malignant cells and tissues.

Huang et al. [200] synthesized a potential brain-targeting gene carrier by conjugating lactoferrin to PAMAM G5 through PEG spacers (PAMAM-PEG-Lf). Lactoferrin is an iron-binding glycoprotein that belongs to the transferrin family. From in vitro transfection studies on primary brain capillary endothelial cells, the transfection efficiency of PAMAM-PEG-Lf-DNA complex was the highest; the luciferase activity of PAMAM-PEG-Lf-DNA complex was about 3- to 3.6-fold higher than that of PAMAM-DNA and PAMAM-PEG-DNA complexes, and about 1.5- to 1.9-fold higher than that of PAMAM-PEG-Tf-DNA (PAMAM-PEG conjugated with transferrin) complex at defined PAMAM to DNA weight ratios. Results from flow cytometry also indicated a higher cellular uptake of the PAMAM-PEG-Lf than PAMAM-PEG-Tf. Consistent results were also obtained from in vivo transfection studies on BALB/c mice; the luciferase activity of PAMAM-PEG-Lf-DNA complex was about 5.2-fold higher than that of PAMAM-DNA and PAMAM-PEG-DNA complexes, and about 2.3-fold higher than that of PAMAM-PEG-Tf-DNA complex within the brain. Fluorescent images of the sections of mouse brain also indicated a higher gene expression mediated by PAMAM-PEG-Lf than PAMAM-PEG-Tf. Interestingly, among the various organs, the gene expression was the most pronounced in the heart, as compared to the liver, spleen, lung or kidney.

4. CONCLUSIONS

Non-viral vectors can be designed in a very biocompatible and non-toxic form with enhancement in physicochemical properties for gene therapy. In this article, we have discussed a variety of modification techniques and cell-targeting strategies to improve chitosan and PAMAM dendrimers for gene delivery. These techniques and strategies are mainly developed based on our fundamental understanding of the mechanisms involved in the cellular uptake and intracellular disposition of the therapeutic genes as well as their carriers. We have seen that the assessment of these non-viral vectors for gene delivery has been largely based on in vitro transfection assays to obtain expression levels as readout. However, this only provides indirect information about the transport properties of these carriers within the cells. Moreover, the contribution of the different uptake pathways involved in polymer-mediated gene delivery has not been well understood and there is still much to learn about the effects of the characteristics of the gene carriers on cellular entry and intracellular trafficking processes. Though these polymers are currently far from being ideal gene carriers, we envision that these polymer-based gene delivery systems will become important tools for gene therapy in the near future.

ABBREVIATIONS

SCID	Severe combined immunodeficiency
CGD	Chronic granulomatous disease
X-CGD	X-linked chronic granulomatous disease
LCA	Leber's congenital amaurosis
PAMAM	Poly(amidoamine)

N/P	Nitrogen to phosphate charge ratio
PEI	Polyethylenimine
TMC	Trimethyl chitosan
TMO	Trimethylated chitosan oligomer
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium sulphate
PEG	Polyethylene glycol
C50	Chitosan 50 kDa
TMC50	TMC 50 kDa
PTMC50	TMC 50 kDa with PEG chains
6ACT	6-amino-6-deoxy-chitosan
IPAAm	N-isopropylacrylamide
DMAEMA	2-(dimethylamino)ethyl methacrylate BMA, Butylmethacrylate
ACS	N-alkylated chitosan
DPPC	Dipalmitoyl-sn-glycero-3-phosphocholine
SA-CSO	Stearic acid grafted chitosan oligosaccharide
GC	Glycol chitosan
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS	N-hydroxysuccinimide
HGC	Hydrophobically modified glycol chitosan
CTAB	Cethyltrimethylammonium bromide
DAMC	Deoxycholic acid-modified chitosan
PLL	Poly(L-lysine)
FBS	Fetal bovine serum
ASGP-R	Asialoglycoprotein receptor
Gal-C	Galactosylated chitosan
Gal-LMWC	Galactosylated low molecular weight chitosan
HMWC	High molecular weight chitosan
LMWC	Low molecular weight chitosan
Gal-C-D	Galactosylated chitosan-graft-dextran
Gal-C-PEG	Galactosylated chitosan-graft-poly(ethylene glycol)
Gal-C-PVP	Galactosylated chitosan-graft-poly(vinyl pyrrolidone)
Man-C	Mannosylated chitosan
mIL-12	Murine interleukin-12
Fol-TMC	Folate conjugated N-trimethyl chitosan
Fol-C	Folate conjugated chitosan
IL-1Ra	Interleukin-1 receptor antagonist

EDA	Ethylene diamine
TAEA	Tris(2-aminoethyl)amine
PAMAM-NHAc	Surface-acetylated PAMAM dendrimer
PAMAM-OH	Hydroxyl-terminated PAMAM dendrimer
QPAMAM	Quaternized PAMAM dendrimer
CyD	Cyclodextrin
PAMAM-Arg	Arginine-grafted-PAMAM dendrimer
PAMAM3-R	Mono-arginine conjugated PAMAM G3
PAMAM4-R	Mono-arginine conjugated PAMAM G4
PAMAM3-R2	Di-arginine conjugated PAMAM G3
PAMAM4-R2	Di-arginine conjugated PAMAM G4
PAMAM-Dexa	PAMAM-dexamethasone
PAMAM-TA	PAMAM-triamcinolone acetonide
PAMAM-TA-L	Low substituted PAMAM-triamcinolone acetonide
PAMAM-TA-H	High substituted PAMAM-triamcinolone acetonide
PAMAM-PEG-Lf	PAMAM-poly(ethylene glycol)-lactoferrin
PAMAM-PEG-Tf	PAMAM-poly(ethylene glycol)-transferrin

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Figure 1.

Repeat units for chitin and chitosan. Chitin consists of mainly n units and chitosan consists of mainly m units distributed in a random fashion.

Figure 2.

Selected examples of chitosan derivatives: (a) Trimethyl chitosan, (b) 6-amino-6-deoxy-chitosan, (c) N-alkylated chitosan, (d) 5β-cholanic acid conjugated glycol chitosan, and (e) Galactose conjugated chitosan.

Figure 3.

Luciferase expression in different cell lines following treatment with C50, TMC50 and PTMC50 polyplexes at different NPR. PEI at NPR of 6 was included for comparison of transfection efficiency. Significance was tested using two-way ANOVA with Bonferroni post test. Two asterisks: $p < 0.01$, three asterisks: $p < 0.001$. Source: [69] (pending the approval of reproduction request from Elsevier).

Figure 4.

The carrier dependent transfection efficiencies measured at media pH 6.5. The HEK 293 cells were incubated with carrier-plasmid DNA complexes for 4 h (with and without FBS) and the transfection efficiencies were observed at 72 h after the transfection. (a) The transfection efficiencies were evaluated by the transfected cell-counting (without FBS). (b) The gene transfections in the presence of 10% FBS. The carrier-DNA weight ratio of chitosan oligosaccharide based carriers was 30 (+/-) and PLL-DNA complex was prepared by the ratio of 2. Data represent mean $\pm$ standard deviations ($n = 4$). Source: [103] (pending the approval of reproduction request from Elsevier).

Figure 5. Structure of PAMAM dendrimer: (a) PAMAM Generation 1, (b) PAMAM Generation 2, and (c) PAMAM Generation 3.

Figure 6.

Selected examples of PAMAM dendrimer derivatives based on PAMAM G3: (a) Activated PAMAM (random hydrolytic cleavage of amide bonds), (b) Quaternized hydroxyl-terminated PAMAM, (c) α -cyclodextrin conjugated PAMAM, and (d) Arginine conjugated PAMAM.

Figure 7.

Transfection efficiency in different cell lines. (a) HepG2 cells, 1.0 μg DNA/well, black bars for (-) FBS, gray bars for (+) FBS. (b) Neuro 2A cells, black bars for 0.2 μg DNA/well, gray bars for 1.0 μg DNA/well. Numbers in parentheses represent charge ratios (+/-). Data represent mean ± standard deviations ($n = 3$). Source: [188] (pending the approval of reproduction request from Elsevier).

Figure 8. Transfection efficiency of PEI, PAMAM, or PAMAM-Dexa into (a) HEK 293 cells and (b) Neuro 2A cells. Transfection efficiency of each complex was measured by luciferase assay. Data represent mean $\pm$ standard deviations ($n = 4$). Source: [191] (pending the approval of reproduction request from Elsevier).