Chapter 12
Functional Dendrimer-Based Vectors for Gene Delivery Applications

Lingdan Kong and Xiangyang Shi

Abstract Poly (amidoamine) (PAMAM) dendrimers are a class of highly branched, monodispersed, synthetic macromolecules with abundant terminal functional groups, and have significant advantages over other cationic polymers as gene delivery vectors due to their well-defined structure, the possibility of facile surface modification, and capacity of carrying large gene segments. The surface amine groups of dendrimers can be conjugated with functional molecules (e.g., hydrophobic moieties, β-cyclodextrin, polyethylene glycol, etc.), and targeting ligands (e.g., folic acid, arginine-glycine-aspartic peptide), while the unique interior of dendrimers affords their uses to form dendrimer-entrapped gold nanoparticles. These modifications render the dendrimer-based vectors with an ability for targeted and enhanced gene delivery, including pDNA and siRNA delivery. In this chapter, we review some recent advances made in multifunctional poly(amidoamine) dendrimer-based nanoparticles for gene delivery applications.

Keywords PAMAM dendrimers · Surface modification · Gold nanoparticles · Gene delivery · Gene silencing

12.1 Introduction

Gene therapy has been considered as a promising approach for cancer therapy due to the fact that exogenous therapeutic genes are able to be delivered to target cells to correct or compensate the genetic defect and abnormality [1–3]. The common

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method of gene therapy including plasmid DNA (pDNA) therapy and small interfering RNA (siRNA) therapy have been intensively studied in treating serious diseases such as cancer and genetic disorders [4, 5]. The foreign gene segments or siRNA delivered into cells could express normal proteins and repair cell defects [4, 6], or destroy the function of cognate mRNA and knockdown the expression of disease-causing proteins, respectively [7, 8]. Although the pDNA and siRNA have been used in gene therapy, the easy enzymatic degradation and limited cell membrane permeability of naked pDNA and siRNA have seriously restricted their in vivo therapeutic applications [9–11]. The success of gene therapy is largely dependent on the development of an ideal delivery system that can selectively and efficiently deliver genetic materials to target cells without causing any associated pathogenic effects [12, 13]. Therefore, the main issue of gene therapy is not the cellular expression of an exogenous gene itself, but the development of safe and efficient gene delivery systems [14].

It is well known that the gene delivery systems include both viral and nonviral vector systems, and viral vectors usually exhibit high transfection efficiency [15]. However, the safety problems raised by the toxicity, oncogenicity, and immunogenicity of the viral vectors greatly hamper their routine use in both basic research laboratories and clinical settings [16–19]. Hence, nonviral delivery systems have continuously received considerable attention because they can be structurally varied, are relatively safe, and have an ability to carry large and diverse genetic materials into cells [14]. Cationic polymers are the commonly used nonviral gene vectors that have been extensively investigated due to their synthetic controllability and multivalent-functionalized surface amine groups, as well as their ability to compact nucleic acid [14, 20–22]. Recent advances in pDNA and siRNA delivery systems including cationic poly-L-lysine, polyethylenimine, diethylaminoethyl-dextran, and chitosan have been proven to be able to transfect genes to different cell lines [21, 23, 24].

Poly(amidoamine) (PAMAM) dendrimers are a class of high branched synthetic macromolecules with narrow molecular weight (Mw) distribution and easily functionalized amine-terminated surface [25–27]. These structural features make PAMAM dendrimer a promising candidate as pDNA or siRNA vector, especially the abundant surface amine groups render PAMAM dendrimers with a strong capacity in compressing pDNA or siRNA to form polyplexes [28–31]. However, some obstacles such as transient gene expression, low transfection efficiency, and high cytotoxicity limit their practical applications [23]. Much effort has been devoted to enhance their gene delivery efficiency and specificity while simultaneously decreasing their cytotoxicity. It has been reported that partial PEGylation [32–35], acetylation [36], alkylation [37, 38], and peptide-conjugation [39, 40] of PAMAM dendrimers can greatly enhance their gene delivery efficiency or specificity, and reduce their cytotoxicity. Therefore, through appropriate surface functionalization of dendrimers, a highly efficient and less toxic nonviral gene delivery vector may be developed for various biomedical applications [41–43]. The aim of this chapter is to give an overview of the recent advances related to the design of functional dendrimer-based vector systems for gene delivery applications.

12.2 Alkylated PAMAM Dendrimers for Gene Delivery

In order to improve the cellular uptake of materials, hydrophobic modification on the surface of dendrimers appears to be crucial, which helps cross the lipid bilayer on the cell surface, thus realizing the safe and efficient delivery of gene. In our previous report, we synthesized hydrophobically modified PAMAM dendrimers by partially reacting the periphery dendrimer amines with 1, 2-epoxyhexane or 1, 2-epoxydodecane [44]. The formed hydrophobically modified generation 5 (G5) dendrimers (denoted as G5.NH_2–C_6 or G5.NH_2–C_{12}) were used to complex two different plasmid DNAs (pDNAs) encoding luciferase (Luc) and enhanced green fluorescent protein (EGFP), respectively, for gene transfection studies.

Cytotoxicity assay data reveal that at a relatively high concentration (above 2000 nM), the cytotoxicity of the vectors follows the order of G5.NH_2–C_{12} > G5.NH_2–C_6 > G5.NH_2. This is likely due to the fact that at a relatively high concentration, the hydrophobic long alkyl chain of dendrimers is able to strongly interact with the hydrophobic cell membrane, thereby enhancing the hole formation on the cell membranes [45].

Subsequently, both Luc and EGFP gene expression assays were used to investigate the gene transfection efficiency of G5 dendrimers in HeLa cells (a human cervical carcinoma cell line). The results showed that in all cases, the Luc gene transfection efficiency decreased with the N/P ratio. And all dendrimeric vectors possessed much higher gene transfection efficiency at the studied N/P ratios than the control cells without transfection treatment and cells transfected with naked pDNA. Under the N/P ratio of 2.5 and 5, the Luc gene transfection efficiency followed the order of G5.NH_2–C_{12} > G5.NH_2–C_6 > G5.NH_2. In particular, at an N/P ratio of 1, the Luc expression value of G5.NH_2-C_{12} was around four times higher than that of G5.NH_2, also much higher than that of the commercial lipofectamine 2000 vector. This suggests that the partial hydrophobic modification of G5 dendrimers enables an enhanced interaction between the vector and cell membrane, thereby significantly enhancing the gene delivery efficiency, in agreement with the literature [37]. However, when compared to the work reported by Santos et al. [37], where the higher gene delivery efficiency was shown by the vectors containing the shortest hydrophobic chains (12 versus 14 or 16 carbon alkyl chains), in this study we show that the gene delivery efficiency of the G5.NH_2–C_{12} dendrimers is much higher than that of the G5.NH_2–C_6 dendrimers. The trend was apparently reversed, although the used numbers of carbon alkyl chains were different.

The gene transfection efficiency of the hydrophobically modified G5 dendrimers was further qualitatively assessed by confocal microscopic imaging of the EGFP gene expression in HeLa cells. At the N/P ratio of 1, all vectors have better gene transfection efficiency than at other N/P ratios. These results were consistent with the Luc assay data. Taken together, both quantitative Luc assay and qualitative EGFP expression confirmed the potential to use both G5.NH_2–C_6 and G5.NH_2–C_{12} dendrimers as vectors for gene delivery applications. Our results suggest that partial

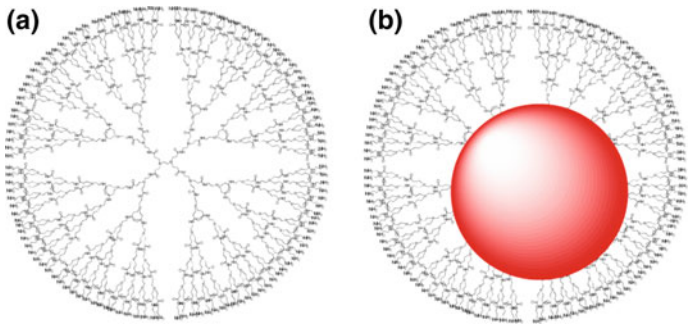
modification of G5 dendrimers with hydrophobic alkyl chains might be an alternative approach to develop dendrimer-based nonviral vectors for enhanced gene delivery applications.

12.3 Au DNEPs for Gene Delivery

Gold nanoparticles (AuNPs) have been identified as a suitable platform for drug/gene delivery due to their unique physicochemical properties, such as size- and shape-dependent optical properties, high surface area to volume ratio, and rich surface chemistry allowing for facile modification with different functionalities [46–52]. Functional AuNPs delivery vehicles, with a positively charged surface, can be used to compress negatively charged genetic materials such as plasmid DNA (pDNA) [42, 53–56] and siRNA [57–61]. It has been reported that lysine dendron-functionalized AuNPs are 28-fold superior to polylysine in reporter gene expression [62]. The super gene transfection performance of the lysine dendron-functionalized AuNPs is likely due to the biomimetic design of the particles that has a size more or less similar to the nucleosome core proteins ($\sim$6 nm) having a large proportion of basic residues (lysine and arginine) that form electrostatic bonding with the phosphate backbone of DNA [63]. In our previous work [42], we designed the use of dendrimer-entrapped Au NPs (Au DENPs) for enhanced gene delivery. The advantages of the Au NP entrapment within the dendrimer interior stem from two aspects: (1) the entrapped AuNPs are able to neutralize some of the dendrimer terminal amines due to the amine stabilization of AuNPs, decreasing the dendrimer cytotoxicity; and (2) the existence of AuNPs helps to reserve the three-dimensional (3-D) spherical shape of dendrimers, thereby significantly improving the DNA or siRNA compaction ability of the dendrimers.

To prove our above hypothesis, amine-terminated G5 PAMAM dendrimers ($G5.NH_2$) were used as templates to synthesize AuNPs with different Au atom/dendrimer molar ratios (25:1, 50:1, 75:1, and 100:1, respectively) [42] (Scheme 12.1). For simplicity, the $G5.NH_2$ dendrimers were denoted as S0, while the Au DENPs with the Au atom/dendrimer molar ratio at 25:1, 50:1, 75:1, and 100:1 were denoted as S25, S50, S75, and S100, respectively, in the naming system.

The gene transfection efficiency of the Au DENP vectors was investigated using firefly Luc gene expression (Fig. 12.1). The gene transfection efficiency is dependent on both the composition of the used Au DENP vectors prepared with different Au atom/dendrimer molar ratios and the selected N/P ratios. The most efficient vector is S25 that has its peak value at an N/P ratio of 2.5:1 for all three different cell lines. For COS-7 cells, the Luc expression reaches 4.27×10^6 RLU/mg protein, while the Luc expression is only 3.42×10^4 RLU/mg protein using S0 as a vector. It appears that at the N/P ratio of 2.5:1, the Luc gene transfection efficiency of the S25 vector is almost 125 times higher than that of the S0 vector. For the other cell lines, at the same N/P ratio, the relative Luc expressed using S25 as a vector is



Scheme 12.1 Schematic illustration of the structures of G5.NH_2 dendrimers (**a**) and Au DENPs (**b**) prepared using G5.NH_2 dendrimers as templates. Reprinted from Ref. [42], Copyright 2012, with permission from Elsevier

153 times (for 293T cells) and 358 times (for HeLa cells) higher than that using S0 as a vector, respectively.

It is interesting to note that the gene transfection efficiency of Au DENPs is not increased with the increase of the Au atom/dendrimer molar ratio, or with the size of the AuNPs entrapped within the dendrimer templates. S25 seems to be the best vector in terms of the Luc transfection efficiency. At the N/P ratio of 2.5:1, both S25 and S50 are able to significantly enhance the gene delivery when compared with S0 without AuNPs ($p < 0.01$ or $p < 0.001$). For the Au DENPs prepared with the increased Au atom/dendrimer molar ratios, the formed AuNP cores have a larger size, which requires more dendrimer terminal amines to stabilize the AuNPs, consequently resulting in a lower binding affinity to pDNA and gene transfection efficiency than those of S25.

Surface modification of dendrimers is reported to cause significant changes in their biological properties, including cytotoxicity and the cell membrane permeation ability [64, 65]. Therefore, it is vital to confirm whether the changes of the physical shape and surface primary amine density of Au DENPs could change the transcellular pathways and localization of the vector by intracellular trafficking and colocalization assay (Fig. 12.2). In the fluorescent microscopic images of cells transfected with all Au DENPs/pDNA complexes, lysosomes were labeled by Lysotracker green (green), Cy3-labeled pDNA (red) were used to localize the polyplexes, and DAPI was used to stain the nuclei (blue). We can see that in the merged images that the polyplexes are well colocalized within the lysosomes and they are all around the nuclei after 2 h of transfection, which means that Au DENPs with different compositions follows the same way as G5.NH_2 dendrimers in the intracellular trafficking pathway and fate.

Results clearly show that Au DENPs with an appropriate composition (Au atom/dendrimer molar ratio = 25:1) enable enhanced gene delivery, with a gene transfection efficiency more than 100 times higher than the G5.NH_2 dendrimers without AuNPs entrapped. It is believed that the entrapment of AuNPs within

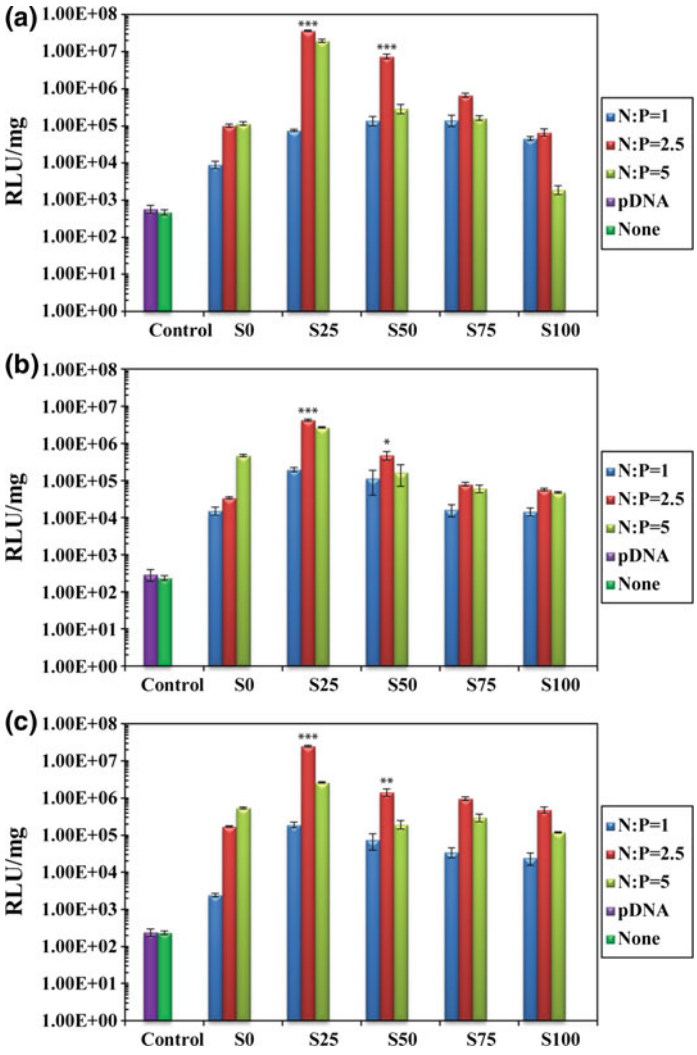


Fig. 12.1 Luciferase gene transfection efficiency of Au DENPs/DNA polyplexes determined in HeLa (**a**), COS-7 (**b**), and 293T (**c**) cells at the N/P ratios of 1:1, 2.5:1, and 5:1, respectively. Transfection was performed at a dose of 1 μg/well of DNA (mean ± SD, $n = 3$). Cells without treatment (None) and cells treated with vector-free pDNA (pDNA) were used as controls. Statistical differences between Au DENPs (S25, S50, S75, and S100, respectively) versus G5.NH_2 dendrimers (S0) at an N/P ratio of 2.5:1 was compared and indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively. Reprinted from Ref. [42], Copyright 2012, with permission from Elsevier

dendrimer templates helps preserve 3-D spherical shape of dendrimers, enabling high compaction of DNA to form smaller particles, and consequently resulting in enhanced gene delivery. With a lower cytotoxicity of Au DENPs than that of the

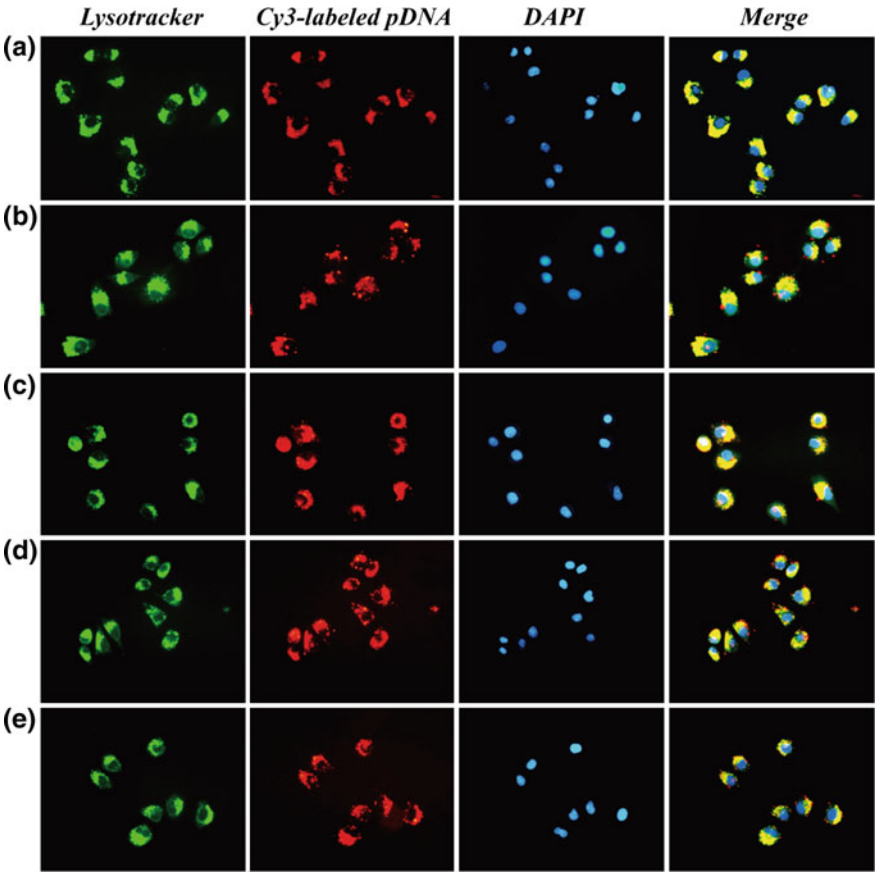


Fig. 12.2 Fluorescence microscopic images (400×) of intracellular trafficking and localization of the Cy3-labeled pDNA with carriers of S0 (**a**), S25 (**b**), S50 (**c**), S75 (**d**), and S100 (**e**) in COS-7 cells recorded after 2 h of gene transfection (*green* Lysotracker green used to label lysosomes; *red* Cy3-labeled pDNA; *blue* DAPI stained cell nuclei). Reprinted from Ref. [42], Copyright 2012, with permission from Elsevier

G5 amine dendrimers and enhanced gene delivery capability, Au DENPs may be used as a new family of gene delivery vectors for various gene therapy applications.

12.3.1 Acetylated Au DNEPs for Gene Delivery

In order to improve the gene delivery efficiency and lower the toxicity, much effort has been devoted to the modification of PAMAM dendrimers. The surface amine groups of dendrimers can be conjugated with hydrophilic polymers [64, 66, 67], or modified with small molecules, such as acetic anhydride [7], cell specific ligands

[68], and amino acid [69–71]. It has been proven that after surface modification, the cytotoxicity of PAMAM dendrimers can be dramatically reduced due to the decreased density of positive charges [34, 35, 37, 38, 72]. Moreover, surface modification may impact the gene transfection efficiency of dendrimers. For example, Waite et al. showed that a modest acetylation (approximately 20%) of PAMAM dendrimers was able to maintain the siRNA delivery efficiency comparable to the unmodified dendrimers, and increased degree of acetylation resulted in the reduced siRNA delivery efficiency [31]. In addition, PAMAM dendrimers modified with L-arginine have been proven to be able to improve the gene delivery efficiency [69, 70]. These studies imply that via an appropriate surface modification, PAMAM dendrimers are able to be afforded with enhanced gene delivery efficiency and simultaneously have reduced toxicity.

Our previous work has shown that Au DENPs prepared using amine-terminated G5 PAMAM dendrimers as templates display higher gene transfection efficiency than G5 dendrimers without the entrapment of AuNPs [42]. To eliminate the possible cytotoxicity resulting from the amine groups on the surface of Au DENPs for safe gene delivery applications, here we synthesized a series of partially acetylated Au DENPs and systematically evaluated the effect of the partial acetylation on their performance in gene transfection. Although acetylation of dendrimer terminal amine groups has been proven to be an effective way to decrease the cytotoxicity of dendrimers, a high degree of acetylation modification may result in reduced gene delivery efficiency due to the decreased density of amine groups left on the dendrimer surface [31]. Therefore, the molar ratios of acetic anhydride to dendrimer were controlled in a range of 5:1–30:1.

The cytotoxicity of partially acetylated and non-acetylated Au DNEPs was evaluated by MTT viability assay of HeLa cells treated with the vectors at different concentrations. It could be seen that all vectors did not have apparent cytotoxicity at low vector concentrations (500 nM or below). With the increase of vector concentration, the cell viability gradually decreased; however, the partially acetylated Au DENPs displayed much less cytotoxicity than non-acetylated ones due to the decreased surface positive charge, in agreement with the literature [7]. We also note that at the high vector concentrations (1000–3000 nM), the cell viability increases with the acetylation degree of the Au DENPs vectors, and $\{(Au^0)_{25}\text{-G5.NH}_2\text{-Ac}_{30}\}$ with the highest acetylation degree exhibits the lowest cytotoxicity at all the tested concentrations. Compared with amine-terminated $\{(Au^0)_{25}\text{-G5.NH}_2\}$, $\{(Au^0)_{25}\text{-G5.NH}_2\text{-Ac}_{30}\}$ caused a significant increase in cell viability at the concentration of 2000 nM ($p < 0.01$). At the highest vector concentration of 3000 nM, partially acetylated Au DENPs (both $\{(Au^0)_{25}\text{-G5.NH}_2\text{-Ac}_{20}\}$ and $\{(Au^0)_{25}\text{-G5.NH}_2\text{-Ac}_{30}\}$) show significantly improved cell viability when compared with $\{(Au^0)_{25}\text{-G5.NH}_2\}$ ($p < 0.01$ and $p < 0.001$, respectively). Therefore, the partially acetylated Au DENPs show advantages over non-acetylated ones in gene delivery applications due to their relatively low cytotoxicity.

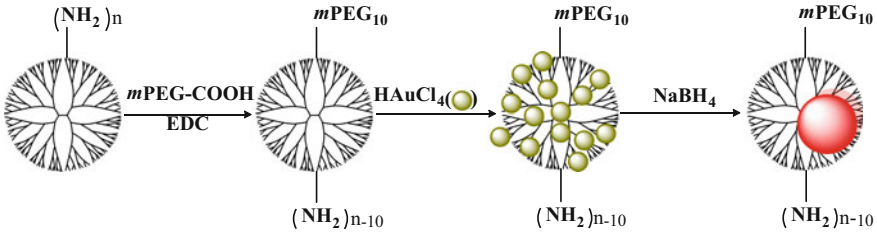
The gene transfection efficiency of the acetylated and non-acetylated Au DENP vectors was investigated by luciferase activity assay after gene expression in HeLa cells. The gene transfection efficiency is largely dependent on the selected N/P ratio,

and it seems that the acetylation degree of Au DENPs did not significantly impact the gene transfection efficiency under the same N/P ratios. All vectors displayed highest Luc activity at the N/P ratio of 2.5:1, therefore this N/P ratio was selected as an optimal one for further study. We believe that the acetylated Au DENPs have advantages in gene delivery due to their less cytotoxicity. Our study suggests that under certain degree of partial acetylation, the reduced amine density on the surface of Au DENPs may slightly compromise the DNA compaction ability, but does not significantly impact the gene delivery efficiency under the optimized N/P ratio (2.5:1). The gene transfection efficiency of acetylated Au DENP vectors was further qualitatively assessed by microscopic observation of the EGFP gene expression. All Au DENPs vectors enabled high EGFP expression, corroborating the Luc activity assay results. With the proven less cytotoxicity of the partially acetylated Au DENPs than that of non-acetylated Au DENPs by cell viability assay, the developed partially acetylated Au DENPs may serve as promising vectors for safe gene delivery applications with non-compromised gene transfection efficiency.

12.3.2 PEGylated Au DNEPs for Gene Delivery

Polyethylene glycol (PEG) is a class of macromolecules with good biocompatibility, non-immunogenicity, and high antifouling property [72–74]. Previous studies have shown that surface PEGylation could enhance the gene delivery efficiency and reduce the cytotoxicity of PAMAM dendrimers [27, 66, 75]. For example, Qi et al. reported that generation 5 PAMAM (G5.NH_2) or generation 6 PAMAM (G6.NH_2) dendrimers partially conjugated with PEG monomethyl ether (*m*PEG) could dramatically facilitate intramuscular gene delivery in neonatal mice [35]. In another study, Tang et al. [27] showed that intramuscular delivery of GFP-siRNA using PEG-modified G5.NH_2 dendrimers could significantly suppress the expression of EGFP gene in both transient adenovirus infected C57BL/6 mice and EGFP transgenic mice. It is believed that with the PEGylation of dendrimer periphery, both the water solubility of the polymer/DNA polyplexes and the intracellular release of DNA molecules can be enhanced, thereby resulting in enhanced gene delivery efficiency [3, 76]. In addition to the *m*PEG conjugation with terminal periphery amine groups of dendrimers, a rational design towards the interior space of PAMAM dendrimers is also feasible in reducing the cytotoxicity and improving the gene transfection efficiency of dendrimers [28, 42, 43]. In either case of dendrimer surface PEGylation or interior Au NP entrapment, the positive charge of the dendrimer surface amines is able to be compromised, thereby having improved cytocompatibility.

Inspired by the fact that both of the exterior modified *m*PEG and the interior entrapped AuNPs are able to decrease the cytotoxicity and increase the gene delivery efficiency of PAMAM dendrimers, we herein attempted to develop a unique PAMAM-based nonviral gene delivery vector by simultaneously modifying *m*PEG onto the dendrimer surface and entrapping AuNPs within the dendrimer



Scheme 12.2 Schematic illustration of the preparation of partially PEGylated Au DENPs. Reproduced from Ref. [77] by permission of The Royal Society of Chemistry

interior (Scheme 12.2) [77]. The pDNA/siRNA transfection efficiency was optimized by altering the molecular weight of *m*PEG (Mw = 2 K or 5 K) and the gold salt/dendrimer molar ratio (25:1 or 50:1) of the Au DENPs. The partially PEGylated Au DENPs were employed to deliver Luc reporter gene, EGFP gene, or B-cell lymphoma-2 (Bcl-2) siRNA to HeLa cells in vitro. The major point of this work is to utilize Au DENPs with different PEGylation chain lengths and different Au contents to explore their efficacy in pDNA and siRNA delivery.

Similar to the previous characterization methods, the viability of cells treated with the G5.NH_2 dendrimers was lower than 60% at a concentration at 3000 nM, while cells treated with the partially PEGylated Au DENPs showed a viability higher than 80% at the same concentration. DNA transfection efficiency of all vectors was investigated by Luc activity assay. Compared with HeLa cells treated with the G5.NH_2 dendrimer/pDNA polyplexes or PEGylated G5.NH_2 dendrimer/pDNA polyplexes, the Luc expression of HeLa cells treated with the partially PEGylated Au DENPs/pDNA polyplexes was remarkably increased (Fig. 12.3a). It could be seen that partially PEGylated Au DENPs/pDNA polyplexes showed the highest Luc expression at the N/P ratio of 5:1, and {$(Au^0)_{50}$-G5.NH_2-*m*PEG2K} vector enabled the highest Luc expression within cells at various N/P ratios. At the N/P ratio of 5:1, the average light unit of {$(Au^0)_{50}$-G5.NH_2-*m*PEG2K}-transfected HeLa cells was 7.39×10^7 RLU/mg protein, which was 292 times higher than that of cells transfected with the G5.NH_2 dendrimers (2.53×10^5 RLU/mg protein). Therefore, the N/P ratio of 5:1 was used for further study. Undoubtedly, {$(Au^0)_{50}$-G5.NH_2-*m*PEG2K} possesses a significantly higher Luc gene transfection efficiency than Au DENPs without PEGylation modification, implying that both the exterior surface *m*PEG modification and the interior Au NP entrapment significantly contribute to the enhanced gene delivery efficiency. The DNA transfection efficiency of all vectors at the optimal N/P ratio of 5:1 was further qualitatively validated by confocal microscopic observation of the EGFP gene expression (Fig. 12.3b). Clearly, there was no obvious EGFP expression in cells treated with PBS, naked pDNA or G5.NH_2/pDNA complexes. In sharp contrast, HeLa cells incubated with all PEGylated Au DENPs/pDNA polyplexes showed a remarkably increased EGFP expression. The confocal microscopic observation further indicates that {$(Au^0)_{50}$-G5.NH_2-*m*PEG2K} vector has excellent

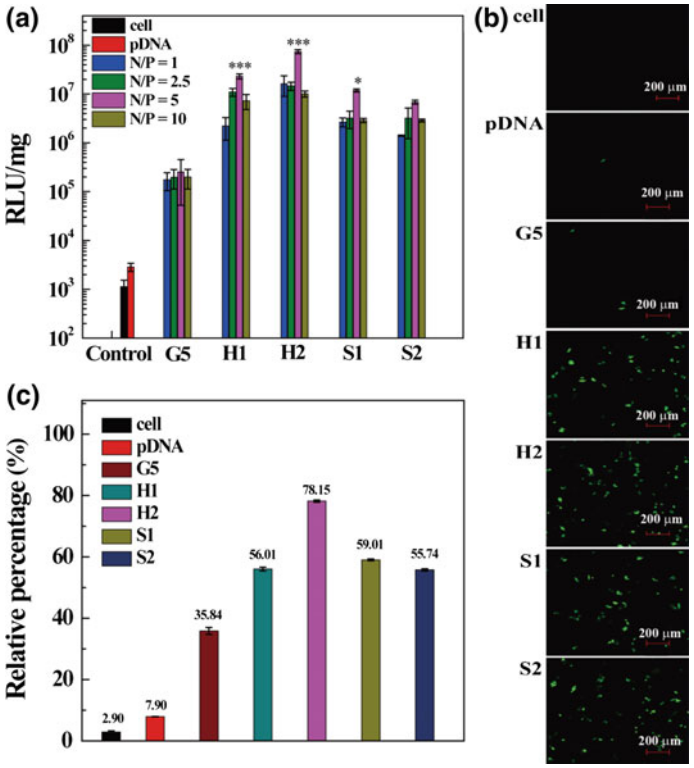


Fig. 12.3 Gene transfection and intracellular uptake efficiency of different vector/pDNA polyplexes in HeLa cells. **a** Luc gene transfection efficiency at N/P ratios of 1:1, 2.5:1, 5:1 and 10:1, respectively (mean ± SD, $n = 3$). Statistical differences between PEGylated Au DENPs/pDNA polyplexes (H1, H2, S1, and S2, respectively) versus G5.NH_2/pDNA polyplexes at an N/P ratio of 5:1 was compared. **b** Confocal microscopic images of HeLa cells treated with different vector/EGFP-pDNA polyplexes at the N/P ratio of 5:1. **c** Flow cytometry measurement of HeLa cells incubated with different vector/Cy3-labeled pDNA polyplexes at the N/P ratio of 5:1 for 2 h. Transfection was performed at a dose of 1 μg/well of DNA (mean ± SD, $n = 3$). Cells without treatment (cell) and cells treated with vector-free pDNA (pDNA) were used as controls. The denotations are as follows: H1 for $\{(Au^0)_{25}$-G5.NH_2-*m*PEG2K$\}$, H2 for $\{(Au^0)_{50}$-G5.NH_2-*m*PEG2K$\}$ S1 for $\{(Au^0)_{25}$-G5.NH_2-*m*PEG5K$\}$, and S2 for $\{(Au^0)_{50}$-G5.NH_2-*m*PEG5K$\}$, respectively. Reproduced from Ref. [77] by permission of The Royal Society of Chemistry

gene transfection efficiency, in accordance with the quantitative Luc activity assay results.

Likewise, cellular uptake capability of the polyplexes was necessary for enhanced gene delivery and expression. Cy3-labeled pDNA was selected as a probe to compare the cellular uptake of different PEGylated Au DENPs/DNA polyplexes using flow cytometry (Fig. 12.3c). It can be found that about 78.15% HeLa cells display Cy3-derived red fluorescence signal when $\{(Au^0)_{50}$-G5.NH_2-*m*PEG2K$\}$ was used as the vector, while the populations of the red fluorescent cells are 56.01,

59.01, and 55.74%, when {$(Au^0)_{25}$-G5.NH_2-*m*PEG2K}, {$(Au^0)_{25}$-G5.NH_2-*m*PEG5K}, and {$(Au^0)_{50}$-G5.NH_2-*m*PEG5K} were used as the vectors, respectively. As expected, HeLa cells treated with all of the synthesized vectors show a higher population of red fluorescent cells than those treated with the G5.NH_2 vector (35.84%).

Under the optimized conditions, the use of the partially PEGylated Au DENPs as vectors for siRNA delivery was explored. Bcl-2 siRNA was able to knockdown the expression of the Bcl-2 protein, a class of anti-apoptotic defense protein related to the multiple drug resistance in cancer cells. In this study, Bcl-2 siRNA was chosen as a model siRNA to evaluate the siRNA delivery capacity of the partially PEGylated Au DENPs. The siRNA transfection efficiency was finally comparatively studied by western blot analysis of Bcl-2 protein expression (Fig. 12.4). The Bcl-2 protein expression level in HeLa cells treated with G5.NH_2/siRNA polyplex was set at 100%. Clearly, similar to Luc gene delivery, HeLa cells treated with {$(Au^0)_{50}$-G5.NH_2-*m*PEG2K}/Bcl-2 siRNA polyplex displayed the lowest Bcl-2 protein expression (15%). The Bcl-2 protein expression levels are 56, 40, and 26% when {$(Au^0)_{25}$-G5.NH_2-*m*PEG2K}, {$(Au^0)_{25}$-G5.NH_2-*m*PEG5K}, and {$(Au^0)_{50}$-G5.NH_2-*m*PEG5K} were used as the vectors, respectively.

These results prove that both of the interior entrapped AuNPs and exterior conjugated PEG chains can enhance the gene transfection efficiency of the G5.NH_2

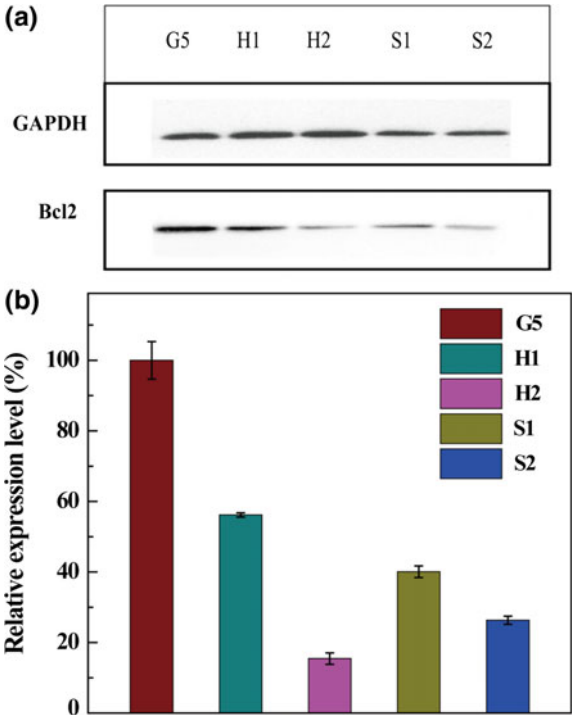


Fig. 12.4 Transfection efficiency of different vector/siRNA polyplexes in HeLa cells. **a** Protein expression levels of Bcl-2 detected by western blot assay. **b** The quantification of relative expression level (%) of Bcl-2 relative to GAPDH (mean ± SD, $n = 3$). The expression of Bcl-2 in G5.NH_2/Bcl-2 siRNA-treated HeLa was set to be 100%. Reproduced from Ref. [77] by permission of The Royal Society of Chemistry

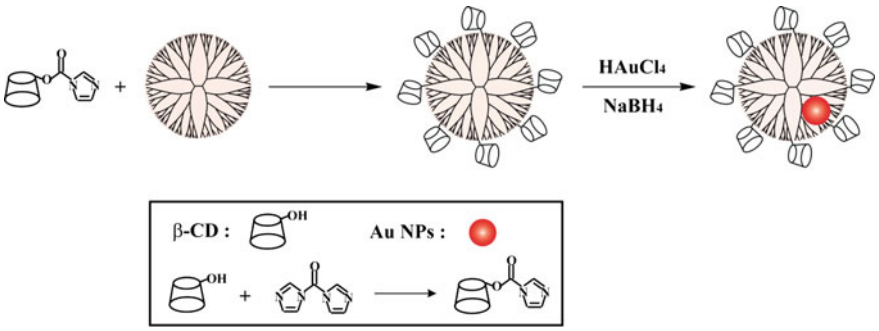
dendrimers. It is interesting to note that the proton sponge effect originated from the tertiary amine groups of PAMAM dendrimers or Au DENPs plays a key role in the endosomal escape of the vector/gene complexes [42, 78]. Although the dendrimers were subjected to surface partial PEGylation modification and interior Au NP entrapment, the proton sponge effect is believed not to be compromised and still makes the vector/pDNA or vector/siRNA polyplexes escape from the endosomes. Overall, $\{(Au^0)_{50}$-G5.NH_2-*m*PEG2K$\}$ is demonstrated to be the best vector for enhanced DNA or siRNA delivery among all the PEGylated Au DENPs.

12.3.3 β-CD-Modified Au DENPs for Gene Delivery

It is well known that cyclodextrins (CD) possess low toxicity [79, 80], non-immunogenicity and excellent hydrophobic cavity [81–83]. CD has been used as an enhancer in both viral and nonviral oligonucleotide delivery due to its binding with nucleic acids, facilitating increased gene stability against nuclease [84–86]. Meanwhile, positively charged CD has been explored as a new vector for gene delivery [87, 88]. Furthermore, various polycations covalently linked with CD have improved biocompatibility, water solubility, and gene transfection efficiency [89]. The enhanced gene delivery efficiency using CD-conjugated polymers could be due to the possible mechanisms that CD-polymer conjugates might increase the release of pDNA or the pDNA complex from endosomes following cellular uptake, although it is still unclear to what degree the membrane disruptive ability of CD-polymer conjugates contributes to the enhancing effect on gene transfer activity [85]. For instance, Gonzalez et al. [90] synthesized β-cyclodextrin (β-CD)-containing polymers for enhanced gene delivery applications, and showed that β-CD was able to be used as a biocompatibility- and solubility-enhancing moiety. It is reasonable to hypothesize that β-CD-modified G5 PAMAM dendrimers with AuNPs loaded within their interior may be developed as a highly efficient gene delivery vector.

In Qiu et al.'s study [91], an approach to using β-CD-modified dendrimer-entrapped AuNPs (Au DENPs) for enhanced gene delivery applications was presented (Scheme 12.3). G5.NH_2-β-CD was first synthesized by modification of G5.NH_2 dendrimer with N,N'-carbonyldiimidazole-activated β-CD according to the literature [92]. The gene transfection efficiency of the Au DENPs-β-CD vector was evaluated by transfecting both Luc reporter gene and EGFP gene to 293T cells in vitro.

According to the characterization of ^{1}H NMR, the number of β-CD molecules conjugated on each G5 dendrimer was calculated to be 8.4, which decreased the cytotoxicity of G5 dendrimers to some extent. The cytotoxicity of the materials follows the order of Au DENPs-β-CD < Au DENPs < G5.NH_2. Subsequent gene transfection experiment proved that at the N/P ratios of 5:1 and 10:1, the Luc expression using Au DENPs-β-CD vector reached 6.2×10^6 and 5.9×10^6 RLU/mg, respectively, significantly higher than that using G5.NH_2 and Au DENPs



Scheme 12.3 Schematic representation of the synthesis of Au DENPs-β-CD. Reproduced from Ref. [91] by permission of The Royal Society of Chemistry

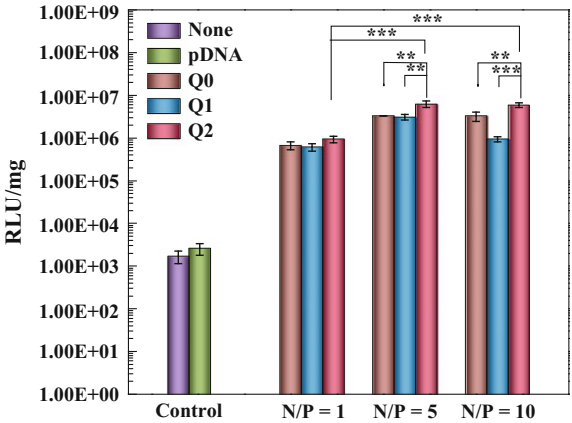


Fig. 12.5 Luc gene transfection efficiency in 293T cells transfected using Q0 (G5.NH_2), Q1 (Au DENPs), and Q2 (Au DENPs-β-CD) vectors at the N/P ratios of 1:1, 5:1, 10:1, respectively. Cells without treatment were used as controls. Data are shown with mean ± SD (n = 3). Reproduced from Ref. [91] by permission of The Royal Society of Chemistry

vectors ($p < 0.01$) (Fig. 12.5). The result of EGFP expression was in accordance with the above data, showing the enhanced gene transfection efficiency of the Au DENPs-β-CD. Overall, our results imply that the surface conjugation of β-CD and the interior entrapment of AuNPs render the G5 dendrimers with less cytotoxicity than Au DENPs without β-CD conjugation, and enable more efficient cellular gene delivery than Au DENPs without β-CD conjugation.

12.4 Targeted Au DENPs for Gene Delivery

In order to realize targeted gene delivery, it is necessary to modify the dendrimer surface with a targeting ligand that can target to cells specifically via a receptor-mediated manner. Various targeting ligands such as folic acid (FA) [56, 93], peptides [28, 94], antibodies [95, 96], lactobionic acid (LA) [97, 98], and

hyaluronic acid (HA) [99, 100], can be modified onto the surface of dendrimers for gene delivery. In this part, we focus on some recent developments of the Au DENP-based vectors for targeted gene delivery applications.

12.4.1 FA-Targeted Au DENPs for Gene Delivery

As one of the most studied cancer-targeting ligands, FA has been known to target FA receptors (FAR) that are overexpressed in several human carcinomas including breast, ovary, endometrium, kidney, lung, head, and neck, brain, and myeloid cancers [101–103]. In our study, we reported the use of FA-modified Au DENPs (Au DENPs-FA) for targeted gene delivery applications [43]. First, amine-terminated G5 dendrimers (G5.NH_2) were covalently modified with FA. The formed FA-modified G5 dendrimers (G5.NH_2-FA) were then used as templates to synthesize Au DENPs-FA. For comparison, Au DENPs without FA were used as a control.

Interestingly, Au DENPs-FA seemed to be less toxic than Au DENPs especially at high concentrations. This was presumably due to the fact that the surface modification of FA moieties onto the dendrimer surface might be able to alleviate the strong electrostatic interaction between the particles and the cells, although both vectors were measured to have a similar number of primary amines on the particle surfaces.

We used both firefly Luc and EGFP gene expression assays to investigate the targeted gene transfection efficiency of Au DENPs-FA vector in FAR-overexpressing HeLa cells. Figure 12.6a shows the gene transfection efficiency of both Au DENPs and Au DENPs-FA vectors as a function of the N/P ratio. It can be seen that both vectors have much higher transfection efficiency than that of the control cells without transfection treatment and cells transfected with naked pDNA, however, the transfection efficiency of both vectors is lower than that of the lipofectamine 2000 vector. We also show that the modification of FA onto Au DENPs affords Au DENPs-FA with much higher gene delivery efficiency compared to Au DENPs vector without FA at the N/P ratios of 1, 2.5, and 5 ($p < 0.001$). The gene transfection efficiency of both vectors is largely dependent on the N/P ratio of the polyplexes. At the N/P ratios of 2.5 and 5, the Luc expression value for Au DENPs-FA vector reached 2.19×10^6 and 1.99×10^6, respectively, significantly higher than that of Au DENPs without FA and the N/P ratio of 2.5 gave rise to the highest Luc transfection efficiency for the FA-targeted Au DENPs. These results suggest that the FA-modification onto the Au DENPs is able to render the vector with significantly high gene transfection efficiency to FAR-overexpressing HeLa cells, although both Au DENPs with or without FA display no appreciable difference in the binding affinity to pDNA. It should be noted that the entrapment of Au core NPs within dendrimers is essential to have improved gene delivery efficiency. Using G5.NH_2-FA dendrimers without the core AuNPs as a vector, the gene delivery efficiency is much less than that using the Au DENPs-FA vector.

This further suggests that the entrapment of Au core NPs are able to render the 3-D spherical shape of dendrimers, significantly improving the gene delivery efficiency, in agreement with our previous results [104].

The targeted gene transfection efficiency of Au DENPs-FA vector was also confirmed by EGFP expression assay. In this case, the gene delivery efficiency was determined by flow cytometry and reported as mean fluorescence intensity, which represents the amount of the EGFP expression. As shown in Fig. 12.6b, at the N/P ratios of 2.5 and 5, the Au DENPs-FA vector have significantly higher gene delivery efficiency than Au DENPs vector without FA-modification, corroborating with the Luc transfection assay data. Our results clearly suggest that the developed Au DENPs-FA vector was able to specifically deliver genes to FAR-overexpressing cancer cells via a receptor-mediated targeting pathway.

12.4.2 RGD-Targeted Au DENPs for Gene Delivery

The cell surface integrin receptors which are overexpressed in many types of cells including human mesenchymal stem cells (hMSCs), glioblastoma cells, ovarian cancer cells, and breast cancer cells [105–107] have been shown to have a high affinity to bind arginine-glycine-aspartic (Arg-Gly-Asp, RGD) peptide [35]. Therefore, RGD peptide has been identified as a promising targeting ligand for different biomedical applications [108]. Dendrimers modified with RGD peptide have been demonstrated to have binding specificity to integrin-overexpressing cancer cells for drug delivery [109, 110] and specific gene delivery [40] applications.

In one of our studies, we developed the use of RGD-modified Au DENPs for highly efficient and specific gene delivery to stem cells, where G5 dendrimers

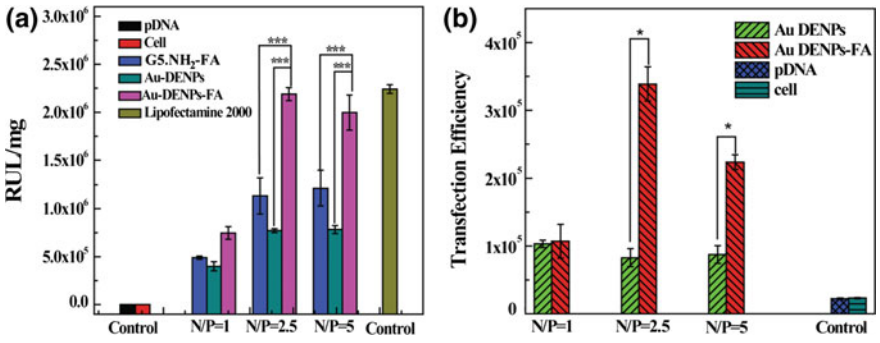


Fig. 12.6 **a** Luciferase gene transfection efficiency of Au DENPs/pDNA and Au DENPs-FA/pDNA polyplexes determined in HeLa cells at the N/P ratios of 1:1, 2.5:1, and 5:1, respectively. **b** EGFP gene transfection efficiency of Au DENPs/pDNA and Au DENPs-FA/pDNA polyplexes determined in HeLa cells at the N/P ratios of 1:1, 2.5:1, and 5:1, respectively. Reproduced from Ref. [43] by permission of The Royal Society of Chemistry

modified with RGD via a poly(ethylene glycol) (PEG) spacer or with PEG monomethyl ether were used as templates to entrap AuNPs [28]. The RGD-modified PEGylated dendrimers and the respective well characterized Au DENPs were used as vectors to transfect hMSCs with pDNA carrying both the EGFP and the luciferase (pEGFPLuc) reporter genes, as well as pDNA encoding the human bone morphogenetic protein-2 (hBMP-2) gene.

In spite of the modification of PEG and RGD moieties onto the surface of dendrimers and internal entrapment of AuNPs, the hydrodynamic diameter (approximately 150–200 nm) and surface potential (around 20 mV) of the particles are appropriate for gene delivery. The prepared RGD-modified PEGylated Au DENPs own better biocompatibility than other groups in the given concentration range. This may be attributed to the fact that the modification of G5.NH_2 dendrimers via surface PEGylation or interior AuNPs entrapment is beneficial to improve the cytocompatibility of the dendrimer-based vectors. In vitro gene transfection efficiency of pEGFPLuc reporter genes demonstrated by quantitative Luc activity assay and qualitative evaluation by fluorescence microscopy revealed that PEG-RGD-modified Au DENPs could transfer pEGFPLuc DNA to hMSCs successfully and possessed the highest delivery efficiency at an N/P ratio of 2.5.

Based on the transfection performance of pEGFPLuc, we selected an N/P ratio of 2.5:1 to evaluate the possibility of using the developed vector systems to transfect hMSCs with a pDNA carrying the hBMP-2 reporter gene. From Fig. 12.7a, it was clear that the non-transfected cells did not have appreciable hBMP-2 expression. In contrast, the hBMP-2 protein was expressed in cells transfected with the different dendrimer-based vectors. The hBMP-2 gene transfection efficiency followed the order of $\{(Au^0)_{25}$-G5.NH_2-$(PEG\text{-}RGD)_{10}$-$mPEG_{10}\}$ DENPs (K4) > $\{(Au^0)_{25}$-G5.NH_2-$mPEG_{20}\}$ DENPs (K2) > G5.NH_2-$(PEG\text{-}RGD)_{10}$-$mPEG_{10}$ dendrimers (K3) > G5.NH_2-$mPEG_{20}$ dendrimers (K1) > G5.NH_2 dendrimers, similar to the above pEGFPLuc gene transfection results. The highest hBMP-2 gene transfection efficiency using the K4 vector should be due to the fact that the PEG-RGD modification of dendrimers and the entrapment of AuNPs within the dendrimers render the vector with RGD-mediated targeting effect and well-maintained 3-D conformation of dendrimers, respectively.

To confirm the hBMP-2 gene transfection-induced stem cell osteogenic differentiation, the activity of ALP, which was a membrane-bound enzyme secreted early in bone formation and had been identified to be an important early marker of osteogenesis [2, 35, 111], was analyzed. Figure 12.7b showed that the ALP activity increased with the cell culture time for hMSCs transfected with a given vector/pDNA complex. Additionally, the ALP activity of hMSCs on day 21 was significantly higher than that on days 7 and 14. At all time points, the ALP activity for the transfected hMSCs was obviously higher than that for the non-transfected cells. K4 displayed the highest ALP activity when compared with the other vectors due to the surface PEG-RGD modification and the entrapped AuNPs.

Osteocalcin was synthesized and secreted by osteoblasts, and had been identified as an important marker of late-stage osteogenic differentiation [112]. Both non-transfected and transfected cells displayed a low production rate of osteocalcin

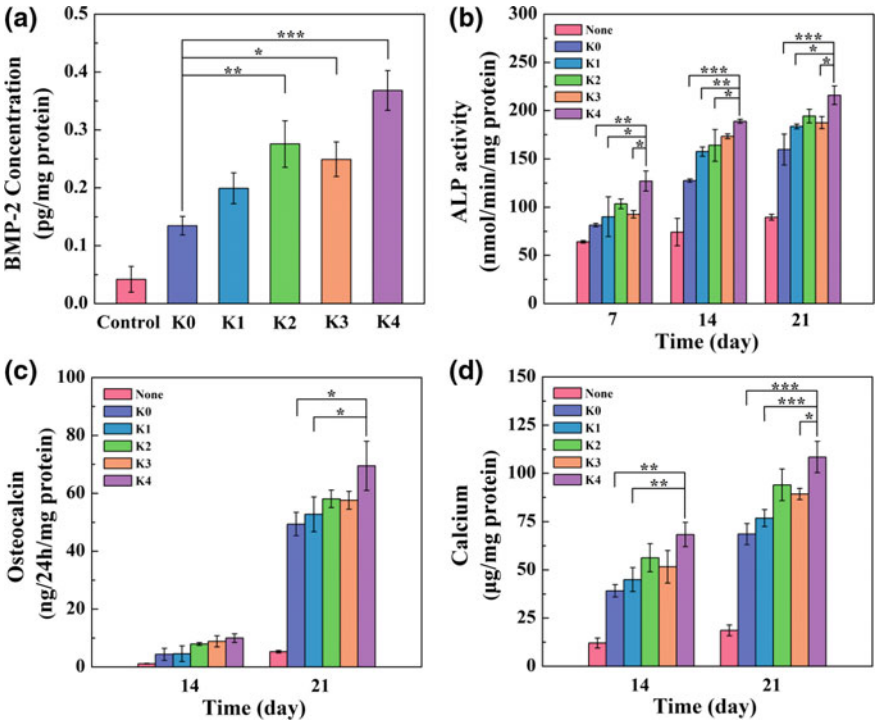


Fig. 12.7 **a** hBMP-2 expression in hMSCs 3 days post-transfection via different vectors; **b** Time course of ALP activity of hMSCs transfected with different vector/pDNA polyplexes; **c** Osteocalcin content secreted by hMSCs transfected with different vector/pDNA polyplexes at different culture times; **d** Calcium deposition on the extracellular matrix of the hMSCs transfected with different vector/pDNA polyplexes after being cultured for 14 and 21 days. All data were presented as mean ± SD (n = 3). Non-transfected cells were used as control. Reprinted with the permission from Ref. [28]. Copyright 2015 American Chemical Society

on day 14 (Fig. 12.7c). On day 21, a distinct increase in the level of osteocalcin secretion was detected for all transfected hMSCs, suggesting that hMSCs after transfection with the hBMP-2 gene using different dendrimer-based vectors are able to differentiate into the osteoblast lineage. Also, relative to G5.NH_2 dendrimers, other four vector-transfected hMSCs were all observed to have visibly higher levels of osteocalcin secretion on day 21. Similarly, the K4 enabled the transfected hMSCs to have the highest osteocalcin secretion when compared to all other vectors, corroborating the ALP activity data.

Calcium deposition was another indicator that could be used to further characterize the degree of hMSCs osteogenic differentiation (Fig. 12.7d). The results clearly demonstrated higher quantities of calcium production in the transfected hMSCs cultures than in the non-transfected cultures. With time, the deposition of calcium progressively increased from day 14 to day 21, in agreement with work previously reported in the literature [113]. On both day 14 and day 21, hMSCs

transfected by the K4 vector displayed the highest calcium content when compared to the other vectors. These results further verified that all dendrimer-based vectors can deliver exogenous genes and successfully induce the hMSCs osteogenic differentiation, with K4 being the most effective vector. Our study suggests that the gene delivery efficiency is largely dependent on the composition and surface modification of the vector. The surface modification with PEG-RGD and the entrapment of AuNPs render the dendrimer platform not only with targeting specificity to recognize integrin-expressing hMSCs, but also with the well-maintained 3-D conformation to have improved DNA compaction ability, thus affording the dendrimer-based vector with high gene transfection efficiency and specificity.

12.5 Conclusion and Outlooks

This chapter gives a brief literature overview of the recent advances in the use of dendrimer-based nanopaticles for gene delivery. To realize enhanced and specific gene delivery applications, dendrimers can be surface modified via alkylation, acetylation or PEGylation, can be surface modified with targeting ligands (e.g., FA, RGD peptide, and can be entrapped with AuNPs.

Although much effort has been devoted to the development of dendrimer-based NPs for gene delivery, this area of research still remains largely unexplored and needs to be further expanded. In view of the abundant amine groups on the dendrimer surface, antineoplastic drugs (e.g., doxorubicin or paclitaxel) may be conjugated, achieving combinational gene therapy and chemotherapy, significantly enhancing the efficacy of cancer therapy. In addition, due to the excellent antifouling property of zwitterionic materials, it is believed that further modification of zwitterionic materials onto the surface of dendrimers may be an effective approach to improve or optimize the transfection efficiency of the materials. Moreover, Au DENPs possess CT imaging effect when the molar ratio of Au/dendrimer achieves the certain proportion, hence, the combination of CT imaging and gene therapy is worthy of development. Besides, various photothermal agents may be modified with Au DENPs, thereby realizing the combinational photothermal therapy and gene therapy. Overall, All these challenges will stimulate scientists to develop multifunctional dendrimer-based NPs for gene delivery and other biological applications.

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