Gene delivery

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Lipid–Gold-Nanoparticle Hybrid-Based Gene Delivery**

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Gene delivery holds promise to cure many inheritable or acquired diseases that are currently considered incurable.\cite{1–7} Liposomes are clinically well-established drug-delivery vehicles with promising properties, including biocompatibility, high drug-to-carrier ratios, and straightforward drug-encapsulation capability.\cite{8–11} However, widespread clinical use of this delivery system still requires better delivery efficiency and lower cytotoxicity. It is a daunting task to develop the optimum delivery system with minimum toxicity and maximum delivery efficiency because toxicity and delivery efficiency often increase or decrease simultaneously. Recently developed nanomaterial-based delivery schemes, which enable precise physical and chemical control of nanoscale structures, hold great promise in addressing these problems.\cite{12–21} In particular, it has been demonstrated that gold nanoparticles (AuNPs) serve as excellent vehicles for the delivery of genetic materials into cells.\cite{22–26} A gene-delivery system based on AuNPs would be especially attractive because the particles can be readily conjugated with targeting biomolecules\cite{27} as well as genes at high packing densities, and this high packing density facilitates high delivery efficiency and low enzymatic degradation of modified DNA strands. Moreover, AuNPs are neither highly toxic nor strongly immunogenic.\cite{28,29}

Here, we devise lipid-DNA-AuNP (L-DNA-AuNP) hybrid-based gene delivery system for better transfection efficiency and reduced cytotoxicity by taking advantages of both AuNPs and liposomes as gene-delivery vehicles (Figure 1). DNA strands loaded onto AuNPs are supported and stabilized, and more DNA strands are delivered per delivery event. These factors enhance transfection efficiency. However, negatively charged DNA strands are exposed in this form; this negative charge deteriorates the contact between DNA-AuNPs and cells because the cell membrane is also negatively charged. This could be overcome by attaching a cationic lipid layer to DNA-modified AuNPs. Not only could this cationic lipid layer (outer layer) facilitate cell membrane crossing of DNA-modified AuNPs, but it could also protect modified DNA.

In a proof-of-concept experiment, we first modified plasmid DNA (pDNA) strands to AuNPs. Our conjugation strategy was based on electrostatic modification of pDNA directly with AuNPs at relatively high pH (pH 9), and subsequent modification of these gene-modified AuNPs with lipid vesicles for the eventual preparation of L-pDNA-AuNP hybrids (Figure 1). First, citrate-stabilized AuNPs were centrifuged, and the supernatant was removed. Next, pDNA (200 ng in 10 mM phosphate buffer at pH 9) was added to the centrifuged NPs. Negatively charged phosphates of the pDNA chain should act as polydentates and facilitate replacement of citrates by pDNA. This substitution reaction is possible because the bonding energy between Au and phosphates (in pDNA) is higher than that between Au and carboxylates (in citrates).\cite{30} For all experiments, the same amount of pDNA was used (200 ng in 10 mM phosphate buffer at pH 9). We used 15-nm, 30-nm, 50-nm, and 80-nm AuNPs (Ted Pella, Inc., Redding, CA, USA) to prepare these hybrids. The amount of added AuNPs was determined such that the same total surface area was available for each AuNP system, regardless of the nanoparticle size (please see Experimental Section for details). To determine the amount of loaded pDNA per particle, UV/Vis spectrophotometry (Agilent Technology, Inc., Waldbronn, Germany) was used (pDNA has UV/Vis absorption at 260 nm; see the Experimental Section for details). We used a supernatant AuNP solution (without loading pDNA) as the blank, because AuNPs also exhibit UV absorption at 260 nm. The number of pDNA per AuNP for each size was obtained by dividing the number of added pDNA by the number of nanoparticles. The number of pDNA per particle was proportional to the calculated surface area per particle, as shown in Figure 2a. The results show that, using this straightforward electrostatic modification process, AuNPs carry 10–100 pDNA strands per particle depending on their size (Figure 2a). Zeta potential measurements further proved that the AuNPs were well modified with pDNA (the increased negative charge of the complex is due to the highly negative charged pDNA; see Fig. S1 in Supporting Information). The high DNA-loading capacity of the AuNPs should improve pDNA delivery efficiency without delivering excessive amount of pDNA. Moreover, because the AuNPs are densely modified with pDNA strands, they are less likely to be degraded by cell nuclei.\cite{31,32} The addition of cationic liposomes to pDNA-AuNP complexes might minimize or entirely remove the negative charge exposure of pDNA, and help deliver pDNA-AuNP complexes through cell membranes with a slight negative charge. Moreover, AuNP platforms should give these lipid complexes further structural stability. Further, lipid vesicles help minimize nonspecific adsorption of biomolecules, including immunogenic plasma proteins (e.g., antibodies), protect genes on AuNP, facilitate cell transfection, and improve targeting specificity by incorporating cell-specific ligands.

Next, pDNA-AuNPs were modified with cationic liposomes to form L-pDNA-AuNP hybrids. Salts in the medium could induce defects in liposomes. We believe defective...
cationic lipid vesicles interact with anionic pDNA-AuNPs to form lipid layers around pDNA-AuNPs. The pDNA-AuNP solution (200 ng of pDNA in 10 μl of 10 mM phosphate buffer and 5 μl of concentration-adjusted AuNP) was incubated with cationic liposomes. Fetal bovine serum (FBS)-free Dulbecco's modified Eagle's medium (DMEM) was then added to the L-pDNA-AuNP solution. The resulting solution was vigorously mixed with a micropipette and incubated at 25 °C for 20 min (for the preparation of lipid-pDNA complexes, 3 μl cationic liposomes (100 ng μl⁻¹) was added in 32 μl FBS-free DMEM was added; for L-pDNA-AuNP preparation, 1.5 μl cationic liposomes (100 ng μl⁻¹) in 33.5 μl FBS-free DMEM was added). The structures of the L-pDNA-AuNP hybrids were then characterized using a cryo-transmission electron microscope (cryo-TEM), which is suitable to verify nanostructures in aqueous environment (please see Supporting Information for experimental details). Two primary structures (type I and type II) were observed in the cryo-TEM images, and more type II hybrids were found than type I (Figure 2b). Because these lipid head groups are cationic and pDNA-modified AuNPs are anionic, we expected encapsulating lipids to form a lipid layer around pDNA and lipids. The cryo-TEM images suggest that the lipid layer in the type I hybrid gets wider and loose upon AuNP contact. We believe type I hybrids are the precursors of type II. Zeta potential data further substantiates the formation of L-pDNA-AuNP complexes (with a decreased negative charge of the complex due to the positive charge provided by cationic lipids please see Fig. S1 in Supporting Information). The exposure of negative charge on the pDNA-modified particle surface induces negative zeta potential. After adding cationic lipids to these particles, however, a dramatic decrease in negative charge occurs, and is due to decreased negative surface charge by the cationic lipid layer in the hybrid (it still retains its negative charge, partially because both type I and II hybrids coexist in solution).

As a proof-of-concept transfection experiment, plasmid DNA (pEGFP-N1), which triggers enhanced green fluorescent protein (EGFP) expression when transfected, was delivered to mammalian cells with various gene delivery vehicles (see Experimental Section for details). EGFP expression was measured and quantified using a fluorescent microscope (Axiovert 200, Carl Zeiss, Germany). A549 cells (human lung cancer cell line) or HeLa cells (human cervical cancer cell line) were mixed with naked pDNA, liposome-encapsulated pDNA, pDNA-AuNPs, and L-pDNA-AuNPs, respectively. The size of nanoparticle (15 nm, 30 nm, 50 nm, and 80 nm) and the amount of added liposomes (3 μl of liposomes (100 ng μl⁻¹) was added in the case of liposome 300 ng (L300), and 1.5 μl of liposomes (100 ng μl⁻¹) was added in the case of liposome 150 ng (L150); Figure 3) were varied. After 48 h incubation at 37 °C, the EGFP expression-based fluorescence signal was measured and analyzed (Figure 3a and b). When pDNA was delivered without lipids and AuNPs, negligible green fluorescence was detected. In the case of the pDNA-AuNP system without liposomes, transfection was a little improved compared to naked DNA, but the efficiency was still poor. These pDNA-AuNP conjugates were not successfully introduced into cells. This could be due to repulsive interaction between the negatively charged cell membranes...
and the highly negatively charged pDNA-AuNP complexes, and nonspecific aggregation of pDNA-AuNPs (especially for larger particles) in relatively high salt concentrations. Commercially available liposomes (Lipofectamine 2000, Invitrogen, Inc., Carlsbad, CA, USA) were then used for EGFP gene transfection. As can be seen in Figure 3a and b, transfection results were distinctly improved for A549 cells. The results show that transfection efficiency in this case was proportional to the amount of lipids used (∼42% for more lipids (L300) and ∼23% for less lipids (L150); see the first row images in Figure 3a). Finally, L-pDNA-AuNPs were used as transfection complexes (with the AuNP size varied from 15 nm to 80 nm). The transfection results show that this lipid-AuNP hybrid system has much better transfection efficiency than the liposome-based system (Figure 3b). In general, larger AuNPs have better transfection efficiency, due to their higher DNA loading capacity for larger particles, but the trend becomes very weak when the particle diameter becomes larger than 30 nm (52% for 15-nm AuNP, 76% for 30-nm AuNP, 78% for 50-nm AuNP, 79% for 80-nm AuNP). This could be because it is more difficult for larger nanoparticles to enter a cell,[28,29] and also because larger particles may induce more nonspecific particle aggregation. Importantly, unlike a conventional liposome-based delivery system, transfection efficiency in this lipid-AuNP hybrid system was not largely affected by the amount of lipid added (Figure 3b). The results suggest that, in this system, pDNA transfection is mainly facilitated by L-pDNA-AuNP hybrids, most pDNA strands are modified on AuNPs, and extra free lipids do not contribute greatly to pDNA transfection. Similar transfection results with similar trends were obtained when HeLa cells were used as transfection target cells (Fig. S2). We also examined cytotoxicity using cell viability tests (please see Experimental Section for details). In this test, dehydrogenases in viable cells induced a change in color, to orange, of the solution. Nontransfected cells were also tested as a control experiment (100% cell viability). The results show that liposome-based transfection system (lipofectamine 2000 in this case) shows the highest cytotoxicity (cell viability was ≈45%). When cells were transfected with AuNP vehicles, in the absence of lipids, cell viability was 94–98% (Figure 4), and cell viability results were affected little by nanoparticle size. However, these results were expected because pDNA-AuNPs showed very poor transfection efficiency. Importantly, the cytotoxicity of L-pDNA-AuNP hybrids was very low (cell viability was 82–90%), and the results were not largely influenced by the size of AuNPs. The results show that only the L-pDNA-AuNP hybrid-based gene delivery system has both high delivery efficiency and low cytotoxicity. This low cytotoxicity of L-pDNA-AuNPs could be partially due to the high stability of L-pDNA-AuNP hybrids. Because L-pDNA-AuNPs with a lipid protection layer and pDNA-supporting Au substrate are more stable than the liposome-pDNA system, the pDNA in L-pDNA-AuNP hybrid is less likely to be degraded or detached inside or outside a cell, and so pDNA can be delivered to the cell nucleus more effectively without inducing much cytotoxicity. Moreover, as our results (Figure 4) suggest, less lipid is needed in the L-pDNA-AuNP system for efficient pDNA delivery than in the lipid-pDNA system. While it is known that high lipid concentrations could induce cytotoxicity, the well-protected, stable AuNP’s low cytotoxicity has been shown elsewhere.[28,29]

In summary, we synthesized L-pDNA-AuNP hybrids, using electrostatic interactions, for gene transfection. These hybrids were carefully characterized and analyzed using UV/Vis spectrophotometer, zeta potential measurement, and cryo-TEM. The results show that 10–100 pDNA strands were modified for each AuNP, and L-pDNA-AuNP hybrids were successfully formed. Transfection assay results proved that the lipid-AuNP hybrid system has high gene delivery efficiency with very low cytotoxicity, due to the synergistic functions of cationic lipids (protection and facilitation of cell penetration) and AuNPs.
and AuNP (supporting and loading many pDNA) in one hybrid. On the other hand, AuNP-based delivery systems suffer from very low delivery efficiency and liposome-based delivery systems have moderate delivery efficiency with high cytotoxicity. Our results show that lipids in general show higher cytotoxicity than AuNPs. A lipid-pDNA-AuNP hybrid delivery system requires less lipid concentration to achieve low cytotoxicity with higher efficiency than a lipid-only delivery system. This study opens up new avenues for exploring lipid-pDNA-NP hybrid synthesis and the use of such hybrids in gene transfection applications.

**Experimental Section**

**Lipid-AuNP–pDNA Conjugates:** First, plasmid DNA (pEGFP-N1) strands were loaded on AuNPs via electrostatic interaction, at pH 9. Prior to use, the pDNA was diluted to 20 ng μl⁻¹ (in 10 mM phosphate buffer at pH 9), and UV/Vis absorbance at 260 nm was measured to quantify the amount of pDNA in solution. Gold nanoparticles (15–80 nm) were centrifuged for 20 min. The diluted pDNA was added to centrifuged AuNP solution. The solution was mixed vigorously, and the mixture solution was allowed to incubate with shaking (300 rpm on an orbital shaker at 37 °C) overnight. Next, pDNA-loaded AuNPs were centrifuged, and UV/Vis absorbance, at 260 nm for the supernatant, was measured to quantify the amount of free pDNA in the supernatant solution. By comparing UV/Vis absorbance values at 260 nm for pDNA before and after the addition of AuNPs, the amount of pDNA per particle can be estimated. To check the stability of these DNA-modified particles, an aliquot of the solution was tested by increasing salt concentration to 50 mM with 200 mM NaCl. In this process, color change from reddish wine to purple means aggregation of AuNPs.

**Transfection Assays:** Transfection was carried out using as described elsewhere. Cancer cell lines (A549) were cultured in 96-well plates with Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Invitrogen, Inc., Carlsbad, CA, USA) supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS) at an initial density of 1.6 × 10⁴ cells per well. It was about 60% confluent on the day of transfection. After 24 h seeding, the cells were washed with serum-free DMEM, and then 100 μl of the serum-free DMEM was added to each well. Finally, the cells were transfected with pDNA or pDNA-modified complexes. After 5 h incubation at 37 °C (under 5% CO₂), the medium was changed to DMEM supplemented with 10% FBS. All experiments were repeated three times.

**Cell Viability Tests:** Cytotoxicity of each transfection method was evaluated using the Cell Counting Kit (CCK-8, Dojindo lab., Japan). A549 cells were grown at a density of 1.6 × 10⁴ cells per well in 96-well plates in 100 μl DMEM supplemented with FBS. After 24 h seeding, cells were incubated with pDNA, lipid-pDNA, pDNA-AuNP and L-pDNA-AuNP for 48 h, and cell viability assay was carried out. The metabolic activity of the cells was measured using CCK-8 (a sensitive colorimetric assay for the determination of the number of viable cells after transfection) means aggregation of AuNPs.

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**References**


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