Gold Nanoparticles for Nucleic Acid Delivery

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Gold nanoparticles provide an attractive and applicable scaffold for delivery of nucleic acids. In this review, we focus on the use of covalent and noncovalent gold nanoparticle conjugates for applications in gene delivery and RNA-interference technologies. We also discuss challenges in nucleic acid delivery, including endosomal entrapment/escape and active delivery/presentation of nucleic acids in the cell.

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INTRODUCTION

Nucleic acids provide promising tools for therapeutic targets, including pathways and molecules involved in cancer and genetic disorders.[1,2] Plasmid[3] and minivector DNAs[4] can be used to repair defective genes, and small interfering RNA (siRNA)[5] can be used to regulate therapeutically relevant processes. In contrast with small-molecule therapeutics, however, nucleic acids require delivery vehicles for protection from nucleases and other environmental agents and to facilitate entry into the cell.[6]

Nucleic acid delivery vehicles are generally divided into two categories: biological and synthetic vectors. On the biological side, viral vectors provide efficient delivery; however, immunogenicity, carcinogenicity, and inflammation can become an issue for clinical applications.[7–9] Traditional synthetic vectors—including cationic lipids,[10] polymers,[11,12] and dendrimers[13]—have been widely used for intracellular nucleic acid delivery. In practice, several lipid-based vectors (*e.g.,* Transfectam[14] and Lipofectamine[15]) have also been commercialized. However, in spite of their transfection efficacy and ease of large-scale production, nucleic acid delivery using these vectors still has limitations for clinical applications, *e.g.,* low storage stability, lack of targeting efficacy, and limited *in vivo* tracking/monitoring.

Inorganic nanoparticles[16–21] are emerging as synthetic vectors that feature several advantages relative to traditional lipid-based vectors, including tunable size and surface properties, multifunctional capabilities, and the ability to translate the physical properties of the metal core to the delivery vehicle. Gold nanoparticles (AuNPs),[22–26] in particular, serve as attractive materials for nucleic acid delivery applications[23,27–34] due to the following advantages. First, AuNPs can be fabricated in a scalable fashion with low size dispersity.[35–38] Second, functional diversity can be readily achieved by the creation of multifunctional monolayers, allowing multiple functional moieties such as nucleic acids and targeting agents to be placed onto the particle surface.[39,40] Finally, the cytotoxicity,[26,41,42] biodistribution,[43,44] and *in vivo* excretion properties[45–47] can be modulated by regulating the particle size and surface functionality.

In this review, we will focus on the structural design of AuNPs for nucleic acid delivery, highlighting the unique chemical and biological properties of these particles.

STRUCTURE DESIGN

There are two primary strategies for the design of nucleic acid carriers: covalent attachment and supramolecular assembly. Both of these approaches have been used with AuNPs, and each has their own advantages and challenges that we will discuss.

Covalent AuNP conjugates

Synthetic and biological compounds can be anchored onto the surface of AuNPs *via* the strong metal–ligand interaction between sulfur and gold (the S–Au binding).[31] The S–Au interaction is partially covalent (~35%) and mostly electrostatic (~65%). Energy decomposition analysis indicates that gold has greater covalent character with sulfur ligands relative to Cu and Ag.[48] In general, the "S–Au covalent bond" is a widely accepted nomenclature. In this section, we will discuss AuNPs functionalized with thiolated oligonucleotides for nucleic acid delivery.

Covalent attachment of nucleic acids to AuNPs is an effective means of transporting gene-silencing oligonucleotides, where the modification does not inhibit biological activity.[49,50] Small silencing RNA reduces target gene expression in addition to inducing regulation by Argonaute proteins that are associated with target messenger RNA (mRNA) degradation.[51] The application of RNA interference (RNAi) using AuNPs mainly involves delivery of microRNAs (miRNAs) and small interfering RNAs (siRNAs).[52,53] Mirkin *et al.*[54] synthesized a class of polyvalent nucleic acid AuNPs (pNA–AuNPs) by functionalizing AuNPs covalently with thiol-modified oligonucleotides and applied them to siRNA-based gene silencing. The dense shell of oligonucleotides on the surface of these NPs inhibits degradation by nucleases, protecting

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the payload. Surprisingly, cellular uptake of these pNA–AuNPs was quite rapid with >50 different cell lines, even though their strong negative charge would be expected to prevent uptake (see following text).[55,56] Cellular uptake of pNA–AuNPs was strongly dependent on the density of the oligonucleotide on the particle surface, with higher density providing more efficient delivery.[57] These "antisense particles" have higher affinity constants for their complementary nucleic acids than their linear counterparts, a key determinant of gene-silencing efficiency. This increased affinity is consistent with the cooperative binding theory that higher oligonucleotide packing densities lead to an increase in the association constant of DNA with AuNPs.[58]

The mechanism of uptake of the strongly anionic pNA–AuNPs remained an open question for a number of years.[55] A recent study by Mirkin[59] demonstrated that spherical pNA–AuNPs, even at a low concentration, could bind strongly to class A scavenger receptors (SR-A), important receptors for mediating membrane transport. On binding to SR-A, endocytosis occurred *via* a lipid-raft-dependent, caveolae-mediated pathway. This mechanism provides "universal" delivery to diseased cells and healthy cells. Hybridizing monoclonal antibody–DNA conjugates with pNA–AuNPs imparts targeting capabilities to these constructs (**Figure 1a**), providing cell selectivity in uptake and greater gene knockdown in cells that overexpress the target antigen (**Figure 1b–e**).[60]

Mirkin *et al.*[61] have used their "antisense particles" to functionalize AuNPs with mimics of synthetic tumor-suppressive miRNA—miR-205. On interaction with the 3′-untranslated region of target mRNA, these miRNA–AuNPs repressed the expression of miRNA target protein, achieving three times the efficiency of the co-carrier-supported molecular miRNAs. Unlike miRNAs, however, siRNAs are totally paired with target mRNA, resulting in transient silencing of the target gene.[62] The instability of siRNAs caused by enzyme degradation, however, provides a challenge for siRNA delivery. Nagasaki *et al.*[53,63] fabricated 15-nm AuNPs with SH-PEG-PAMA (thiol-bearing polyethylene glycol–poly(2-N,N-dimethylamino)ethylmethacrylate) and

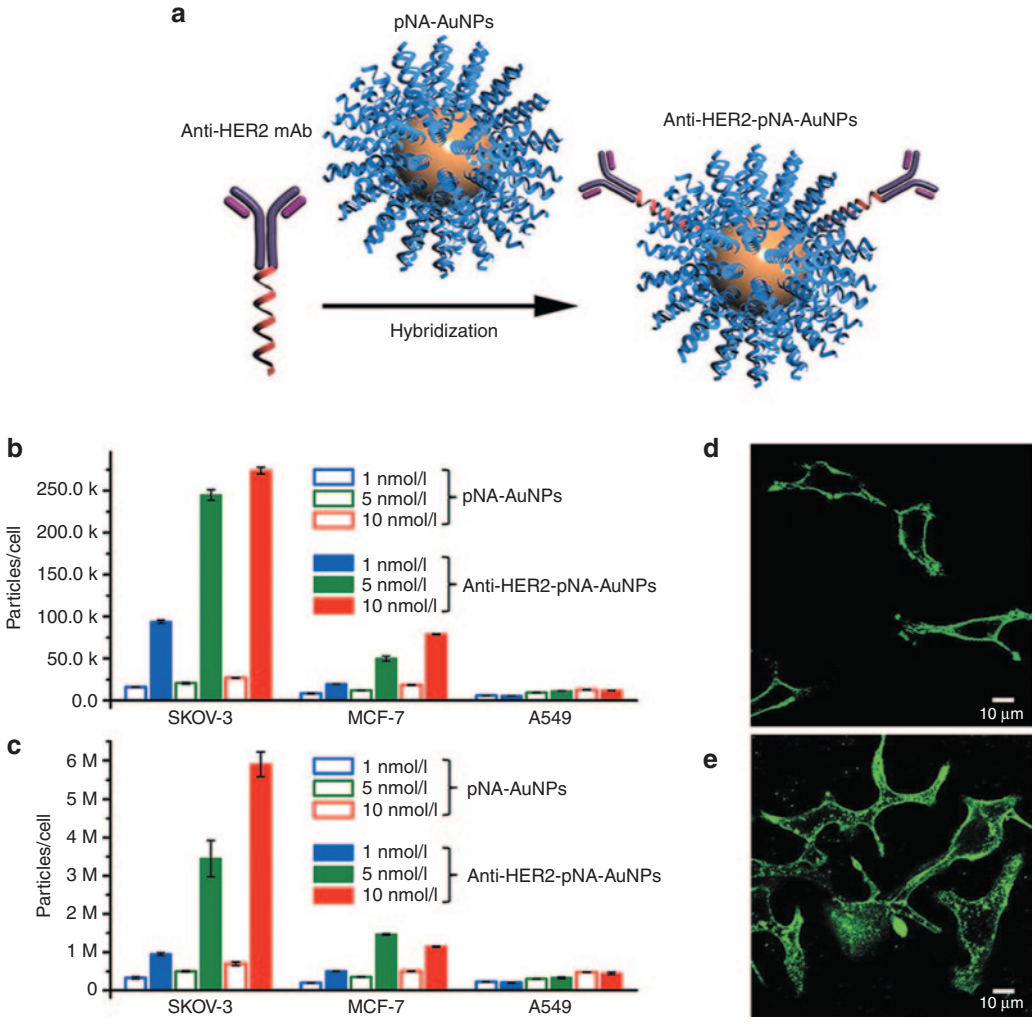


Figure 1 Antibody-linked pNA-AuNPs for cellular targeting. Schematic illustration of (**a**) the synthesis of anti-HER2-pNA–AuNPs. The number of AuNPs per cell at (**b**) 4 °C and (**c**) 37 °C, after 4 hours of incubation. Significantly higher uptake was observed for HER2-overexpressing cells (SKOV-3) and moderate-expressing cells (MCF-7) with regard to targeted particles, whereas HER2-nonexpressing cells (A549) showed no selectivity. Confocal micrograph of SKOV-3 cells after incubating for 4 hours with 5 nmol/l anti-HER2-pNA–AuNPs at (**d**) 4 °C and (**e**) 37 °C. Antisense DNA strands were labeled with fluorescein at the 5′-end. mAb, monoclonal antibody. Reprinted with permission from ref. 60. Copyright 2012 American Chemical Society.

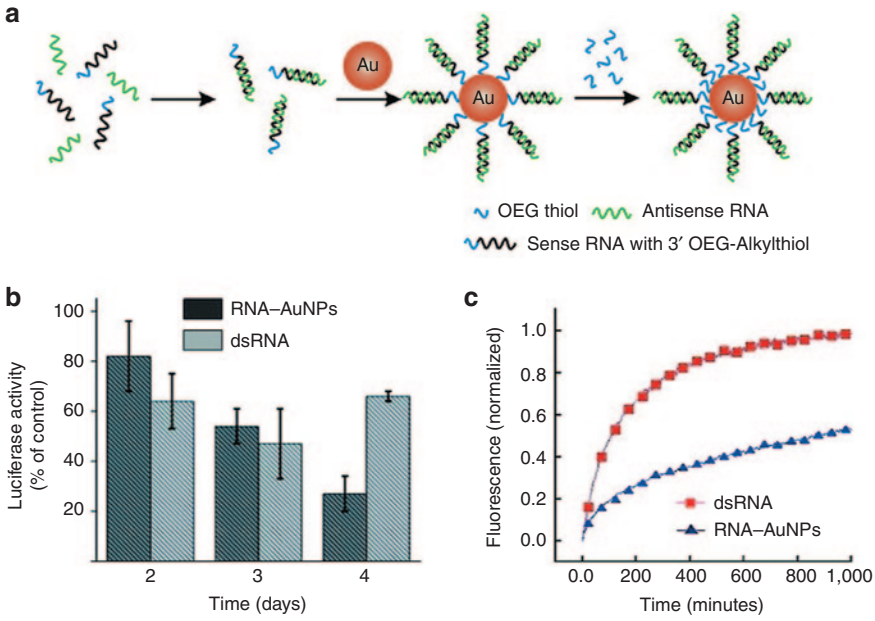
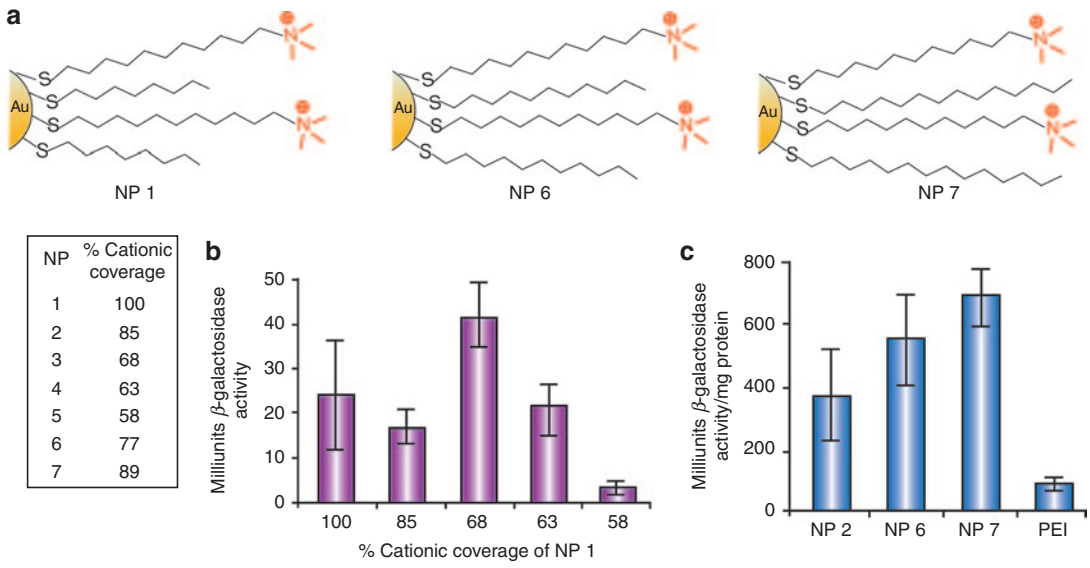


Figure 2 Polyvalent siRNA–AuNPs for gene regulation. (**a**) Schematic depiction of the synthesis of polyvalent RNA–AuNP conjugates. (**b**) Knockdown of luciferase expression in HeLa cells over 4 days using polyvalent RNA–AuNP conjugates (3 nmol/l nanoparticle (NP) concentration, ~100 nmol/l RNA duplex concentration) or double-stranded (ds) RNA (100 nmol/l). (**c**) Stability of RNA–AuNPs. Comparison of the stability of cyanine 5-labeled double-stranded (ds)RNA (red) and RNA–AuNPs (blue) in 10% serum. The increase in fluorescence intensity demonstrates the distance-dependent release of the fluorophore from the gold core, which is an efficient quencher of the fluorescence. Reprinted with permission from ref. 52. Copyright 2009 American Chemical Society. OEG thiol, oligoethylene glycol-thiol.



NP	% Cationic coverage
1	100
2	85
3	68
4	63
5	58
6	77
7	89

Figure 3 Mixed-monolayer-protected AuNPs for gene transfection. (**a**) Structures of cationic AuNPs with various amounts of cationic ligands (NP 1–5) and alkyl chain length (NP 6–7) used for transfection of DNA. (**b**) Transfection of β-galactosidase (β-gal) using NP–DNA complexes at 2,200:1 ratio (measured by β-gal activity). Levels of β-gal were determined by comparison with a standard curve using known protein concentrations. (**c**) Transfection efficiency of NPs 2, 6, and 7 at 2,200:1 NP/DNA ratio and commercially available PEI (60 kDa). NP, nanaoparticle; PEI, polyethyleneimine. Reprinted with permission from ref. 72. Copyright 2002 American Chemical Society.

monothiolated siRNAs, where the amine-terminated copolymer enhances particle stability. Knockdown of ~60% of normalized luciferase expression was observed in HuH-7 cells. In later studies, RNase-free polyvalent siRNA–AuNPs were synthesized and delivered into HeLa cells. These densely siRNA-coated AuNPs had sixfold longer serum half-life than their molecular RNA duplex counterparts (**Figure 2a–c**).[52]

Topical transdermal delivery presents a particularly difficult challenge in therapeutic delivery.[64,65] Recent studies have shown that through the use of appropriate skin conditioning agents, pNA-AuNPs can be used to transdermally deliver siRNA in an effective manner. In these studies, epidermal growth factor receptor was targeted, reducing epidermal thickness by ~40%. Moreover, no clinical or histological evidence of toxicity was found in treated skin.[66]

Although emphasis is often placed on the importance of cellular uptake of nucleic acid delivery systems, endosomal escape is perhaps the most challenging barrier for delivery.[67,68] Recently, siRNA loaded in lipid NPs has shown low efficiency (1–2%) in escaping from the endosomes into the cytosol.[69] The successful intracellular gene regulation by pNA–AuNPs,[52,55,56] however, indicates effective endosomal escape of pNA–AuNPs. Mirkin *et al.*[70] further demonstrated that cyanine 5-labeled pNA–AuNPs gather in endosomes 1 hour posttransfection; after 4 hours, they can be observed throughout the cytoplasm. However, the detailed mechanism regarding endosomal escape of pNA–AuNPs still remains elusive. In addition, cationic polymeric ligands (such as SH-PEG-PAMA) act as stabilizers for AuNPs against RNases and facilitate endosomal escape *via* the "proton sponge" effect. This will be discussed in detail in the next section. Nevertheless, the necessity for nucleic acids to be removed from the NP surface is still an open debate.

Noncovalent AuNP conjugates

Noncovalent nucleic acid delivery vehicles are an attractive alternative to covalent systems. Using supramolecular conjugates allows the use of unmodified nucleic acids, allowing delivery of DNA for gene therapy and of RNA for knockdown applications. With these systems, the synthetic versatility of the AuNP platform provides multiple options for vehicle design, such as mixed-monolayer-protected AuNPs (MM-AuNPs), amino acid–functionalized AuNPs (AA–AuNPs), and layer-by-layer-fabricated AuNPs (LbL-AuNPs).

The strong negative charge of nucleic acids makes cationic AuNPs obvious partners for self-assembly.[71] In early studies, Rotello *et al.*[72] created effective delivery vehicles for plasmid DNA using quaternary ammonium–functionalized AuNPs. These studies demonstrated that a number of parameters were important for successful transfection, including the AuNP-to-DNA ratio, surface charge coverage, and hydrophobicity (**Figure 3a–c**). In these AuNP–DNA complexes, the DNA is bent around the AuNPs into a "spaghetti and meatballs" motif, protecting the DNA from degradation by nucleases and other chemical agents.[73] The changes in DNA conformation were further studied using circular dichroism and fluorescence experiments, demonstrating complex reversible structural changes that occurred on binding.[74] In an analogous strategy, Klibanov and Thomas covalently attached ~2 kDa polyethyleneimine (PEI) chains to AuNPs and used these PEI–AuNP conjugates as delivery vectors of plasmid DNA into mammalian cells.[75] The most potent conjugates were 12 times more efficient at plasmid DNA delivery than their unmodified PEI counterparts.

Amino acids provide versatile moieties for tuning AuNP functionality.[76,77] Binding studies between cationic amino acid–functionalized AuNPs and double-stranded DNA (dsDNA) showed that side chains with increased cationic character were stronger binders than hydrophobic or neutral analogs.[78] This concept was applied to gene delivery, where AuNPs featuring dendritic amino acid–based headgroups were shown to be effective gene delivery vehicles *in vitro*.[79] As an example, first-generation lysine dendron (G1-Lys)–coated AuNPs were 28 times superior to polylysine in reporter gene expression. A similar dendron-based strategy was later used for siRNA delivery, where biodegradable

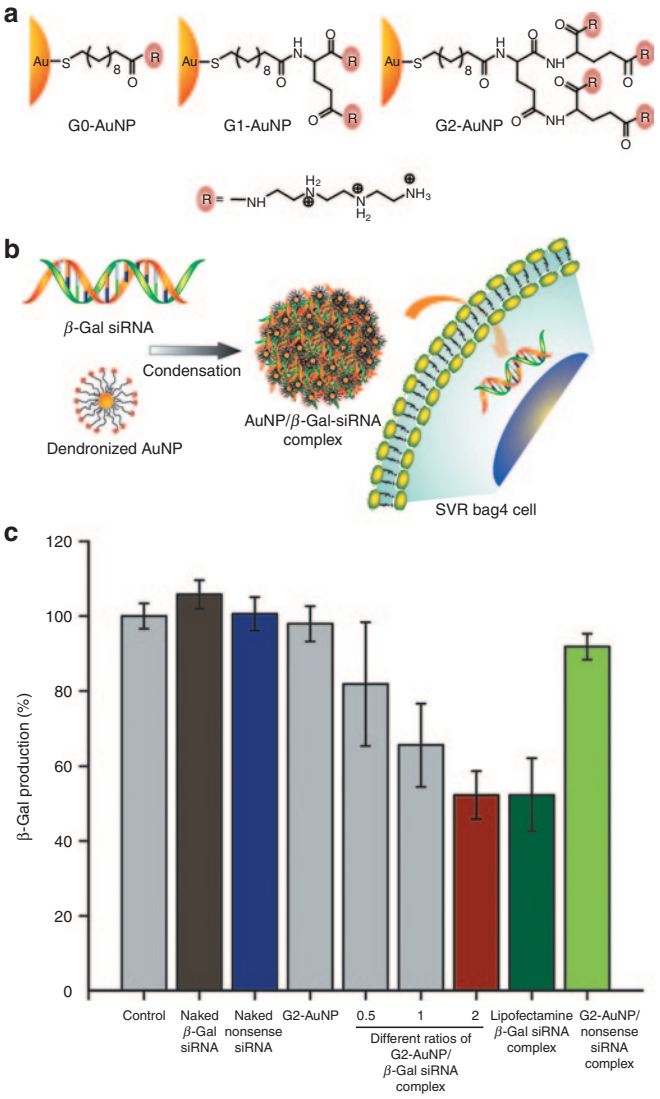


Figure 4 siRNA delivery using dendronized AuNPs. (**a**) Chemical structure of G2-AuNP. (**b**) Schematic illustration of G2-AuNP/β-gal-siRNA complexation and transfection into SVR-bag4 cells. (**c**) Gene-silencing effect of naked *β*-gal-siRNA, naked nonsense siRNA, G2-AuNP, G2-AuNP/*β*-gal-siRNA complex, and G2-AuNP/nonsense-siRNA complex. *β*-gal-siRNA, *β*-galactosidase-siRNA; G2-AuNP, second-generation dendronized AuNPs; NP, nanoparticle; siRNA, small interfering RNA. Reprinted with permission from ref. 80. Copyright 2012 WILEY-VCH KGaA, Weinheim.

glutamic acid scaffolds were functionalized with triethylenetetramine (**Figure 4a**).[80] Second-generation dendronized AuNPs (G2-AuNPs) provided the most efficient knockdown, with *β*-galactosidase–siRNA (*β*-gal–siRNA) (**Figure 4b**) showing ~50% gene silencing with minimal cytotoxicity.

Although measuring the intracellular expression of *β*-galactosidase is an excellent way to investigate the transfection/knockdown efficiency, it does not shed much light on the uptake mechanism and the consequent endosomal release of noncovalent AuNP–NA conjugates. Noncovalent AuNP–nucleic acid (NA) conjugates are in general composed of cationic AuNPs that can facilitate endosomal escape *via* a "proton sponge" mechanism.[67,81] The traditional "proton sponge" hypothesis explains that after fusion of the late endosome with a lysosome, materials (such as

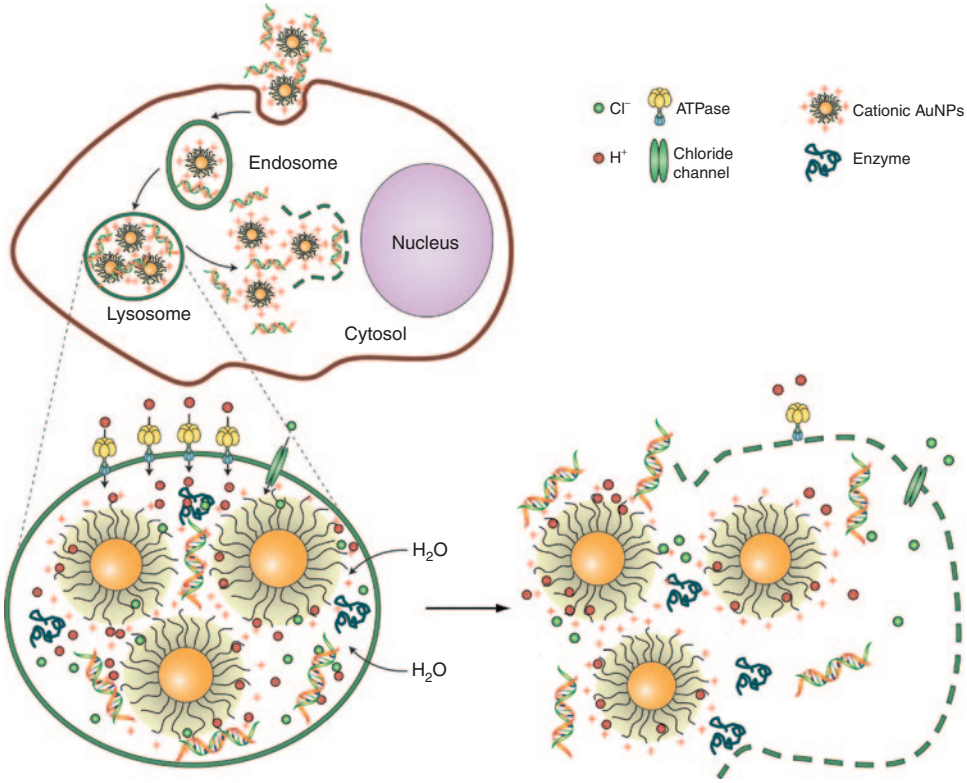


Figure 5 Schematic illustration of the proton sponge effect leading to endosomal escape for cationic nanoparticles. ATPase, adenosine triphosphatase; NP, nanoparticle; PEI, polyethyleneimine. Adapted with permission from ref. 67. Copyright 2009 Macmillan Publishers.

PEI) with amine groups are able to sequester protons once they enter the acidifying lysosomal compartment.[82] The lysosomal "proton pump" process results in the retention of one Cl^- ion and one water molecule for each proton entering the lysosome, maintaining electrical neutrality. This process results in lysosomal swelling and rupture, with materials released into the cytosol (**Figure 5**).[67,83] However, a recent study did not observe any contribution from PEI in lysosomal pH change within a time frame of 0–24 hours, challenging the theory that the "proton sponge" is the dominant releasing mechanism.[84]

LbL fabrication using negatively charged nucleic acids and positively charged AuNPs provides a strategy for the controlled release of nucleic acids. In one example, mercaptoundecanoic acid–stabilized AuNPs were consecutively coated with layers of positively charged PEI and negatively charged siRNAs (**Figure 6a**).[85] Both siRNA/PEI-AuNPs and PEI/siRNA/PEI-AuNPs showed much higher cellular uptake than citrate-stabilized AuNPs. PEI/siRNA/PEI-AuNPs showed nominal toxicity in cell culture despite containing high levels of PEI. For AuNP–NA conjugates constructed by the LbL method, the material on the top layer plays an important role in achieving endosomal escape.[85] In the above example, when siRNAs were used as the outer layer, NPs were packed in the endosome but no enhanced green fluorescent protein knockdown occurred. By contrast, PEI/siRNA/PEI-AuNPs that have PEI on the outside provided 70% knockdown of enhanced green fluorescent protein (**Figure 6b,c**). This comparison indicates an endosomal escape mechanism of siRNA delivery by such LbL-AuNPs.[85] Similarly, AuNPs with an incorporated anionic polyelectrolyte were prepared by an LbL method. Instead of embedding nucleic acids into two PEI layers, *cis*-aconitic anhydride–functionalized poly(allylamine) (PAH-Cit) was enclosed as a charge-reversal layer, with siRNA as the terminal layer (**Figure 6d**).[86] By enhancing the capacity of PEI's "proton sponge" effect, the charge conversion occurring between pH 7.4 and pH 5.0 would facilitate the endosomal escape of these siRNA/PEI/PAH-Cit/PEI–AuNP complexes through membrane disruption. Gene silencing of ~80% was achieved by these complexes, whereas only 20% silencing was observed with controls lacking the charge-reversing element (**Figure 6e**).[86]

STIMULI-RESPONSIVE RELEASE OF NUCLEIC ACID

Glutathione (GSH)-mediated release represents a promising approach for intracellular release of nucleic acids from AuNPs (**Figure 7a**).[79] GSH is the most abundant thiol species in the cytoplasm, with intracellular GSH concentrations (1–10 mmol/l) dramatically higher than the extracellular levels (2 μmol/l in plasma).[87] GSH features an overall negative charge; as a result, place exchange of GSH onto cationic particles diminishes and then reverses particle charge (**Figure 7b,c**). This strategy was hypothesized for the previously discussed dendron-based systems, an assertion validated by a dose-dependent increase in transfection efficiency[79] on treatment of cells with glutathione monoester (GSH-OEt) (**Figure 7d**).[88]

Light-regulated release provides spatiotemporal control of nucleic acid release. In early studies, a photolabile AuNP was designed to photochemically uncage DNA in supramolecular AuNP complexes using near-ultraviolet (>350 nm) irradiation.[89]

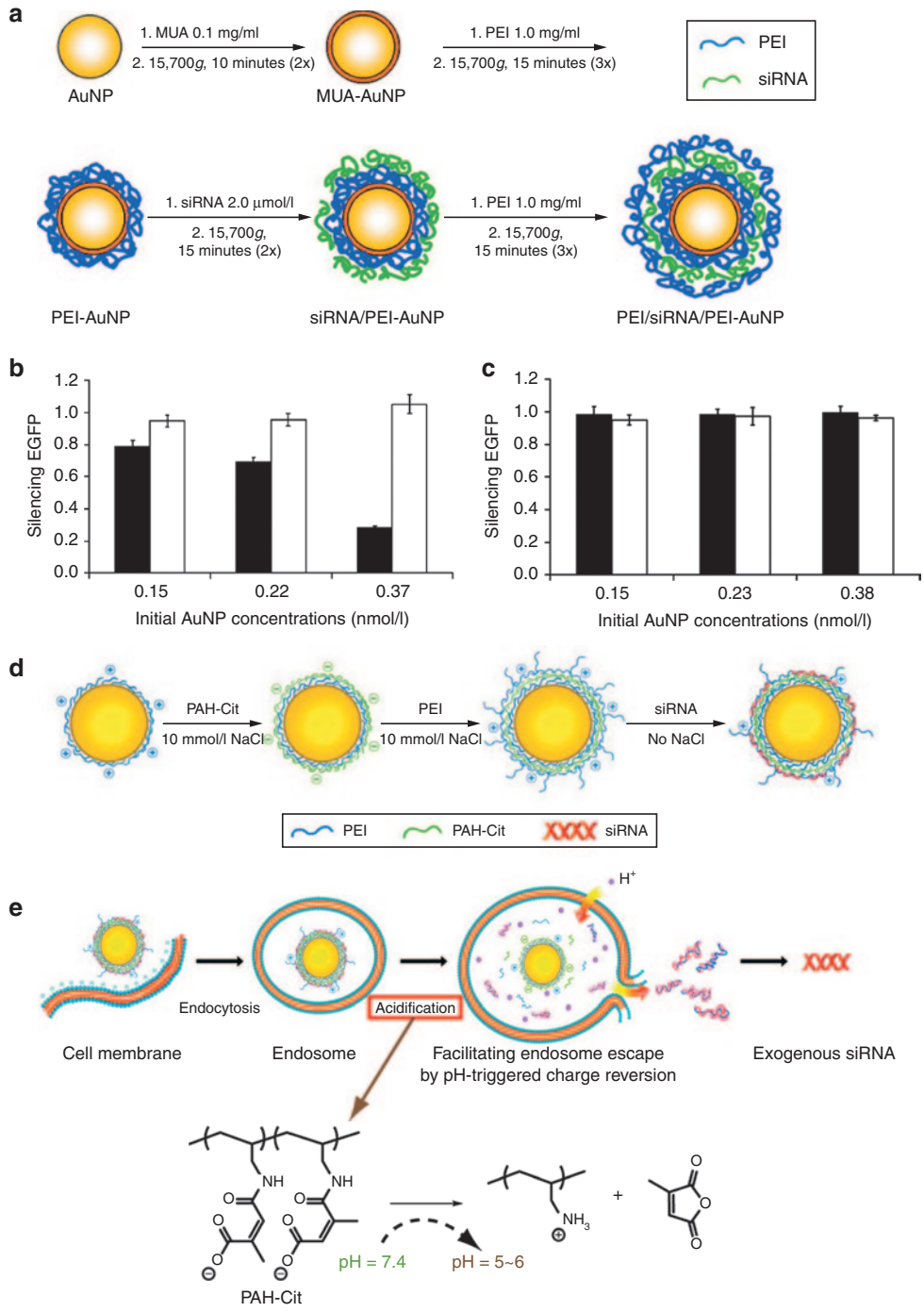


Figure 6 siRNA delivery using LbL-AuNPs. Schematic representation of (**a**) layer-by-layer deposition applied to AuNPs. Gene silencing of enhanced green fluorescent protein (EGFP) in Chinese hamster ovary (CHO)-K1 cells stably expressing EGFP after addition of (**b**) PEI/siRNA/PEI-AuNPs and (**c**) siRNA/PEI-AuNPs at various initial concentrations. For LbL-AuNPs, either siRNA against EGFP (■) was used or a nontargeted siRNA control (□) was used. Reprinted with permission from ref. 85. Copyright 2009 American Chemical Society. Schematic representation showing (**d**) the synthesis of LbL-AuNPs containing a charge-reversal layer (PAH-Cit). Schematic illustration of (**e**) enhanced intracellular payload release at endosome by a pH-dependent layer on AuNPs. LbL-AuNPs, layer-by-layer-fabricated AuNPs; MUA, 11-mercaptoundecanoic acid; NP, nanoparticles; PAH, *cis*-aconitic anhydride-functionalized poly(allylamine); PEI, polyethyleneimine; siRNA, small interfering RNA. Reprinted with permission from ref. 86. Copyright 2010 American Chemical Society.

By constructing a positively charged AuNP bearing a photocleavable *o*-nitrobenzyl ester linkage, the surface net charge of AuNPs could be switched from cationic to anionic under irradiation. The charge reversal yielded a negatively charged carboxylate group and consecutively released negatively charged DNA by electrostatic repulsion (**Figure 8a**). Efficient DNA delivery and release was also obtained in living cells, along with significant internalization of DNA into the nucleus.

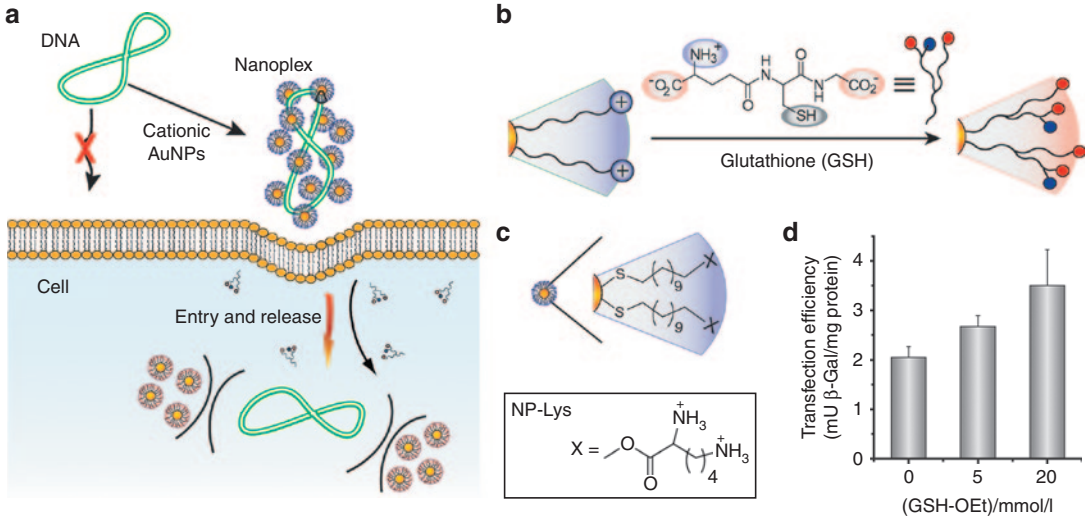


Figure 7 Glutathione-mediated intracellular gene release. (**a**) Schematic illustration of cationic gold nanoparticles (AuNPs) used as transfection vectors. (**b**) Schematic description of place exchange between native cationic ligands and cellular glutathione (GSH) on NP surface. (**c**) Chemical structure of lysine-based functionalized AuNPs. (**d**) Increase in transfection level depending on dose of glutathione monoester (GSH-OEt). Reprinted with permission from ref. 79. Copyright 2008 American Chemical Society.

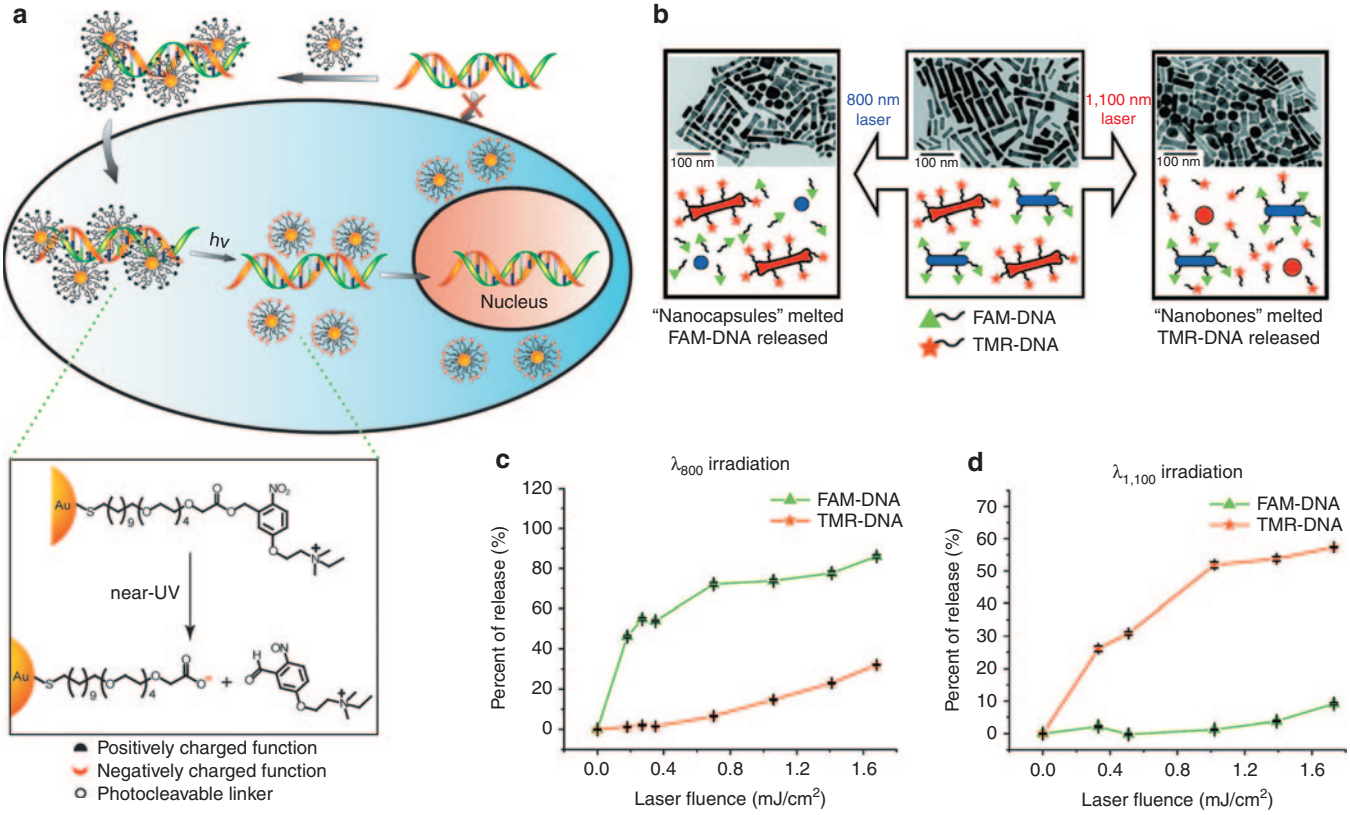


Figure 8 Light-regulated nucleic acid release in cells. Schematic illustration of (**a**) the release of DNA from the AuNP–DNA complexes on ultraviolet irradiation within the cell. Reprinted with permission from ref. 89. Copyright 2006 WILEY-VCH KGaA, Weinheim. Schematic illustration of (**b**) selective release of multiple DNA oligonucleotides from gold nanorods of different aspect ratios. Percentage release of 6-carboxyfluorescein–labeled DNA (FAM–DNA) and tetramethylrhodamine-labeled DNA (TMR-DNA) as a function of (**c**) λ_{800} laser fluence and (**d**) λ_{1100} laser fluence. Reprinted with permission from ref. 93. Copyright 2009 American Chemical Society.

Near-infrared irradiation can be used to broaden the application of light-regulated release, by increasing the depth of tissue penetration up to 10 cm.[90] The strong surface plasmon absorption of gold nanorods (AuNRs) is tunable within the near-infrared range, making these particles excellent platforms for near-infrared applications.[91] These AuNRs can be heated locally by using

pulsed laser excitation, providing release of covalently bound nucleic acids on the rod surface of the AuNRs in a controlled fashion.[92] The longitudinal surface plasmon resonance (SPR_{long}) of AuNRs can be tuned by altering the AuNR aspect ratio, providing a potential mechanism for wavelength-specific release. In one study, AuNRs with two different aspect ratios were functionalized with two different oligonucleotides—6-carboxyfluorescein–labeled DNA and tetramethylrhodamine-labeled DNA (**Figure 8b**). By applying this methodology, selective, externally controlled gene release was achieved by irradiating at 800 and 1100 nm, with a high level of payload delivery (**Figure 8c,d**).[93] However, AuNRs demonstrated low endosomal release. Moreover, their cellular uptake was also lower than the uptake of spherical AuNPs due to their high aspect ratio and consequently lower number of available cell membrane receptor sites for binding.[94] In addition, the difficulty associated with the endosomal and lysosomal escapes of AuNRs is a major concern for transfection efficiency.[95] So far, there is no solid evidence for light-associated endosomal escape.

CONCLUSIONS AND OUTLOOK

Key properties of the AuNPs, such as biocompatibility, tunable size, and straightforward functionalization, make them attractive scaffolds for the creation of nucleic acid delivery vehicles. In particular, the design of AuNP–based covalent and noncovalent nucleic acid carriers significantly affects cellular uptake, endosomal escape, and nucleic acid release. To date, the potential of these systems has been mostly demonstrated using *in vitro* studies; however, there are issues to be resolved before AuNP–NA conjugates can be translated to clinical applications. First, minimizing short- and long-term cytotoxicity of AuNPs is essential. Numerous studies documented the biocompatibility of these therapeutic NPs using simple cytotoxicity experiments; however, detailed toxicological evaluation (cell membrane damage, oxidative stress, genotoxicity, and so on) needs to be properly addressed. Second, targeting of these vehicles to specific organs and tissues will be required to minimize side effects. This targeting can be achieved by (i) decorating the surface of delivery vehicles with specific antibodies targeted to the disease cells and (ii) grafting noninteracting functional groups (*e.g.,* polyethylene glycol and zwitterionic entities) on the surface that eschew plasma protein adsorption, improving the pharmacokinetics and evading immune surveillance. Finally, immunological issues need to be fully explored before clinical use of any new material. Nonetheless, AuNPs provide a platform with all the attributes required to meet these challenges and should continue to provide important tools for *in vitro* applications, eventually yielding clinically important delivery vehicles.

ACKNOWLEDGMENTS

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