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Oligonucleotide-Modified Gold Nanoparticles for Intracellular Gene Regulation

Nathaniel L. Rosi,* David A. Giljohann,* C. Shad Thaxton, Abigail K. R. Lytton-Jean, Min Su Han, Chad A. Mirkin†

We describe the use of gold nanoparticle-oligonucleotide complexes as intracellular gene regulation agents for the control of protein expression in cells. These oligonucleotide-modified nanoparticles have affinity constants for complementary nucleic acids that are higher than their unmodified oligonucleotide counterparts, are less susceptible to degradation by nuclease activity, exhibit greater than 99% cellular uptake, can introduce oligonucleotides at a higher effective concentration than conventional transfection agents, and are nontoxic to the cells under the conditions studied. By chemically tailoring the density of DNA bound to the surface of gold nanoparticles, we demonstrated a tunable gene knockdown.

Nucleic acid-based methods for controlling gene expression have had a considerable impact on research involving gene pathways and function (1–3). In addition, antisense (AS) therapies are potentially powerful candidates for clinical treatments of various ailments, including cancer and HIV/AIDS (1, 4). In conventional AS approaches, oligonucleotides designed to hybridize with target mRNA sequences downregulate the expression of the corresponding protein (EGFP) expressed in C166, a mouse endothelial cell line (ATCC). A phosphorothioate-modified antisense sequence complementary to an internal coding region (bases 1198 to 1215) of the mRNA for EGFP was selected from published literature (22). This sequence was used in the design of two sets of antisense Au NPs, with the ASODN conjugated to the Au NP surface with either one or four thiol groups (Fig. 1). The tetra-thiol particle (particle A) supports 45 to 50 strands, whereas the mono-thiol particle (particle B) has 110 to 120 strands. Whereas particle A exhibits a binding constant to its complementary sequence that is approximately equal to that of an unmodified ASODN, particle B has an affinity ~35 times as high as that of the unmodified ASODN (fig. S1) (23). This result is consistent with a cooperative binding theory, which predicts that higher oligonucleotide packing densities result in a corresponding increase in association constant (15). Taken together, particles A and B offer the opportunity to study the potential of ASNPs in regulating gene expression and, more specifically, the effect of particle binding constants and oligonucleotide loading on the performance of such particles in the context of EGFP expression.

We evaluated the Au NP-ASODN composites (ASNPs) for their cellular uptake, intracellular stability, and the fate of the oligonucleotides once the particles were taken up by the cell. Most approaches to transfection agents rely on the use of positively charged materials to ensure cellular uptake (9, 24). Unexpectedly, ASNPs readily enter cells despite their coating with negatively charged DNA. Entry of the ASNPs into the EGFP-expressing C166 mouse endothelial cells was confirmed by incubating the cells for 48 hours in the presence of ASNPs functionalized with 5′ Cy5.5-labeled

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Fig 1. Preparation of antisense Au NPs. Citrate-stabilized gold nanoparticles (13 nm ± 1 nm) were functionalized with ASODNs that were premodified with an AEt tether and either two cyclic disulfides (DTPA) or an alkyl-thiol anchoring group to produce Antisense Particles A and B, respectively.
ASODNs. The uptake was studied using confocal fluorescence microscopy. Observation of Cy5.5 fluorescence throughout the cytoplasm provided proof of particle uptake. Additionally, we performed identical uptake experiments with various cell types, including RAW 264.7 (macrophage), HeLa (cervical carcinoma), NIH-3T3 (fibroblast), and MDCK (kidney) (fig. S2). In each case, we observed >99% of uptake (virtually every cell incorporated the ASNPs), and there were no differences in cellular morphology as compared with untreated control cells or cell viability under the conditions studied as determined by Trypan Blue staining.

We determined whether the ASODNs remain bound to the gold particle surface within the cell by using “dual fluorophore” ASODNs labeled with both a 3’ fluorophore (Cy3, on the surface of the particle) and a 5’ fluorophore (Cy5.5, at the end of the ASODN) [see (23) for sequence and preparation]. If ASODNs remain stably attached to the particle surface within the cell, both their Cy3 and Cy5.5 fluorescence would be quenched. If they were digested by nucleases, we expected to see free Cy5.5, whereas if they were displaced from the surface of the particle by chemical reduction, we expected to see strong emission from both dyes.

After a 48-hour incubation period in the presence of ASNPs, two-photon confocal laser scanning microscopy of cells (Fig. 2A) with both Cy3 and Cy5.5 fluorescence throughout the cytoplasm provided proof of particle uptake. Additionally, Cy5.5 fluorescence throughout the cell was observed throughout the 48-hour incubation period (virtually every cell incorporated the ASNPs functionalized with 5’ Cy5.5-modified ASODNs. After a 48-hour incubation period, the cells were lysed and the bulk fluorescence of the lysate was measured in the emission range of the Cy5.5 fluorophore (706 to 717 nm). Dithiothreitol (DTT) displacement of the Cy5.5-labeled ASODNs from the nanoparticles resulted in increased Cy5.5 fluorescence emission in the lysate, indicating that many of the ASODNs remain undigested by nucleases after 48 hours within the cellular environment (fig. S4).

Glutathione (GSH) has been used as a reagent to effect DNA release from NPs in the context of gene delivery (26). Intracellular GSH levels differ between various cell types (27), so GSH levels in C166 cells could be lower than other cell lines. However, analysis of the stability of the dual fluorophore-labeled DNA on the Au NP (23) shows that even at elevated GSH levels (10 mM), >60% of the oligonucleotides remain bound to the particle after 48 hours. For cellular systems in which GSH levels are high, it is possible to increase the strength of the interaction of ASODN with Au NPs through the use of cyclic disulfide anchoring groups without substantially changing surface coverage (28, 29). In the case of C166 cells studied, we can conclude from the dual-fluorophore particle stability experiments that the oligonucleotides remain attached to the particle surface.

Given the potential for ASODN degradation by nucleases in vivo, we next examined the in vitro resistance of the ASNP to DNase compared with particle-free oligonucleotides. Fluorescence experiments indicate that particle-bound ASODNs are degraded much slower than are unbound ASODNs (Fig. 2, B and C). The tight packing of the ASODNs on the NP surface likely causes steric inhibition of nuclease degradation. Others have made similar observations in the context of assembly and manipulation of oligonucleotide-Au NP superstructures (30). The increased resistance to nuclease degradation of particle-bound ASODNs should increase the lifetime of the AS agent within the cell.

The activity of the ASNPs with respect to gene knockdown was evaluated with EGFP-expressing C166 cells. Evidence of EGFP knockdown was first observed by two-photon excitation confocal fluorescence microscopy, which indicated that cells treated with ASNPs exhibited noticeably lower fluorescence compared with untreated control cells (Fig. 3). These

**Fig. 2.** Experiments aimed at understanding the intracellular stability of antisense nanoparticles. (A) Fluorescent microscopy images of C166-EGFP cells incubated 48 hours with antisense particle B functionalized with dual-fluorophore labeled ASODNs (3’ Cy3 and 5’ Cy5.5) only reveal fluorescence from Cy5.5 (706 to 717 nm, upper left). Negligible fluorescence is observed in the emission range of Cy3 (565 to 615 nm, upper right). Transmission and composite overlay images are shown in the lower left and lower right quadrants, respectively. The arrows indicate the location of the cell. Similar data collected from experiments using particle A are included in fig. S3 (23). (B) Duplexes composed of either quencher-modified ASODN/fluorophore-modified complement or antisense particle/fluorophore-modified complement were treated with DNase. (C) The ASODN duplex degraded much faster than the antisense particle duplex, as calculated using fluorescence spectroscopy, where \( {F}_{\text{min}} \) is the fluorescence of the mixture at its initial, fully hybridized state and \( {F}_{\text{max}} \) is the maximum fluorescence of the system at 80°C where all of the oligonucleotides are dehybridized.
observations were then confirmed by quantitatively assaying for EGFP expression with fluorescence spectroscopy.

As previously described, the binding properties of the ASNPs can be tailored by controlling the number of ASODNs loaded on the particles. We expected that particle B, with its higher binding affinity, would cause greater reductions in EGFP expression than particle A. To test this hypothesis, samples of particle A were added to C166-EGFP cells. After 48 hours, the cells were collected, lysed, and assayed for EGFP expression (for quantification methods). Noncomplementary ODN-Au NPs (23) were used as controls and showed similar fluorescence levels to nontreated cells (Table 1). Cells incubated with 0.024 nmol or 0.048 nmol of particle A (low binding constant) displayed a 12% and 14% decrease in EGFP expression, respectively. However, when incubated with the same amount of particle B (tight binding constant), the cells displayed a decrease in EGFP expression of 14% and 20%, respectively (Table 1). The differences in the observed knockdown between Particles A and B indicate that the ASNP binding constant can significantly influence the amount of protein expression.

Interestingly, when the particles were compared with commercially available lipoplexes (Lipofectamine 2000, Invitrogen; Cytofectin, Gene Therapy Systems) in the context of EGFP expression, the nanoparticle system outperformed the commercial systems under the conditions studied with respect to percent knockdown, total amount of ASODN delivery, and nontoxicity (Table 1). Following recommended commercial protocols, Lipofectamine and Cytofectin were used to transfect EGFP-C166 cells with an equal

![Image](https://example.com/image.png)

**Table 1.** Performance characteristics of antisense nanoparticles.

<table>
<thead>
<tr>
<th>Antisense experiment</th>
<th>Observed toxicity</th>
<th>Approximate binding constant</th>
<th>Percent decrease in EGFP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense Particles A (0.024 nmol particles 1.08 nmol ASODN)</td>
<td>No</td>
<td>$7.1 \times 10^{-20}$</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Antisense Particles A (0.048 nmol particles 2.16 nmol ASODN)</td>
<td>No</td>
<td>$7.1 \times 10^{-20}$</td>
<td>14 ± 0.4</td>
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<tr>
<td>Antisense Particles B (0.024 nmol particles 2.64 nmol ASODN)</td>
<td>No</td>
<td>$2.6 \times 10^{-22}$</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Antisense Particles B (0.048 nmol particles 5.28 nmol ASODN)</td>
<td>No</td>
<td>$2.6 \times 10^{-22}$</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Nonsense Particles A (0.048 nmol particles)</td>
<td>No</td>
<td>N/A</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>Nonsense Particles B (0.048 nmol particles)</td>
<td>No</td>
<td>N/A</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Lipofectamine 2000 (0.024 nmol ASODN)</td>
<td>No</td>
<td>$6.7 \times 10^{-20}$</td>
<td>6 ± 0.2</td>
</tr>
<tr>
<td>Lipofectamine 2000 (2.64 nmol ASODN)</td>
<td>Yes</td>
<td>$6.7 \times 10^{-20}$</td>
<td>N/A</td>
</tr>
<tr>
<td>Cytofectin (0.024 nmol ASODN)</td>
<td>No</td>
<td>$6.7 \times 10^{-20}$</td>
<td>7 ± 0.7</td>
</tr>
<tr>
<td>Cytofectin (2.64 nmol ASODN)</td>
<td>Yes</td>
<td>$6.7 \times 10^{-20}$</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Preparation of Poly(diiododiacetylene), an Ordered Conjugated Polymer of Carbon and Iodine

Aiwu Sun, Joseph W. Lauher, Nancy S. Goroff*

Conjugated organic polymers generally must include large substituents for stability, either contained within or appended to the polymer chain. In polydiacetylenes, the substituents fulfill another important role: During topochemical polymerization, they control the spacing between the dyne monomers to produce an ordered polymer. By using a co-crystal scaffolding, we have prepared poly(diiododiacetylene), or PIDA, a nearly unadorned carbon chain substituted with only bis(nitrile) oxalamides, aligned by hydrogen bonds between oxalamide groups and weak Lewis acid-base interactions between nitriles and iodoalkynes. In co-crystals with one oxalamide host, the diyne undergoes spontaneous topochemical polymerization to form PIDA. The structure of the dark blue crystals, which look copper-colored under reflected light, has been confirmed by single-crystal x-ray diffraction, ultraviolet-visible absorption spectroscopy, and scanning electron microscopy.

Since Heeger, McDiarmid, and Shirakawa’s discovery of conducting behavior in doped polyacetylene samples (1), carbon-rich molecules and materials have attracted great interest for their electronic and optical properties. Recently examined applications include photovoltaic cells, organic light-emitting diodes (OLEDs), field-effect transistors, and chemical sensors (2–5). Polyaacetylene, the simplest conjugated polymer, consists of a backbone of carbon atoms, each bonded to one hydrogen atom and connected together by alternating single and double bonds. However, this material is thermally unstable and insoluble, making it unsuitable for general use. More manageable conjugated polymers can be prepared from substituted alkynes (6) and other conjugated monomers, such as substituted aromatics; for example, in the commercially useful polymer poly(p-phenylenevinylene) (PPV), the backbone contains benzene rings as well as simple carbon-carbon double bonds. Polyaacetylenes, in which every other double bond in the polyaacetylene backbone is replaced by a triple bond, are potent multiphoton absorbers that have applications in optical limiters, waveguides, and thermometric sensors (7).

Obtaining the desired optical and electronic properties of a given conjugated polymer requires a highly ordered molecular structure. For polydiacetylenes, this presents a substantial synthetic challenge: With solution-phase methods, the monomer dyynes can react in either a 1,2- or 1,4-fashion, leading to a polymer with an irregular, branched backbone instead of regular repeat units. However, as discovered by Wegner (7) and elaborated by Baughman (8), if the dyynes are first aligned appropriately in the solid state at a distance commensurate with the repeat distance in the target polymer, then their arrangement in space will control their reactivity, leading to ordered topochemical polymerization. Chemists have developed many creative approaches to induce appropriate alignment for 1,4-polymerization, not only in the solid state, but also in Langmuir-Blodgett films, liposomes and vesicles, and other ordered chemical interfaces (2, 9, 10). Hydrogen bonding has often

Department of Chemistry, State University of New York, Stony Brook, NY 11794–3400, USA.

*To whom correspondence should be addressed. E-mail: nancy.goroff@sunysb.edu

References and Notes
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