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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 down-regulate the expression of the corresponding translated proteins. Although the potential of AS oligodeoxynucleotides (ASODNs) was recognized more than 20 years ago (5, 6), their development into viable therapeutic systems has faced challenges with regard to stable transfection and entry into diverse cell types, toxicity, and low efficacy (3). Various transfection agents have been developed to shuttle nucleic acids into cells, including cationic lipids and polymers, modified viruses, dendrimers, liposomes, and nanoparticles (1, 4, 7, 8), but each system has particular limitations.

Gold nanoparticles (Au NPs) have a high affinity for biomolecules and have been used as nonviral vectors for DNA delivery (9–13). Gold nanoparticles chemically functionalized with alkylthiol-terminated oligonucleotides (14) are highly stable in saline solutions and bind complementary nucleic acids in a very selective and cooperative manner (15); their equilibrium association constants can be more than two orders of magnitude greater than those observed for unmodified oligonucleotides and their complements (16). These distinctive properties have made oligonucleotide-functionalized Au NPs the centerpiece of several highly sensitive and selective assays for biomolecule detection (17–21). Because of their demonstrated stability and enhanced binding properties, we hypothesized that this particular type of Au NP could efficiently scavenge intracellular DNA or RNA. We introduce the use of oligonucleotide-functionalized Au NPs as new antisense agents that take advantage of the ensemble properties of the nanoparticle-oligonucleotide conjugate.

In initial experiments, we targeted mRNA sequences coding for enhanced green fluorescent 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

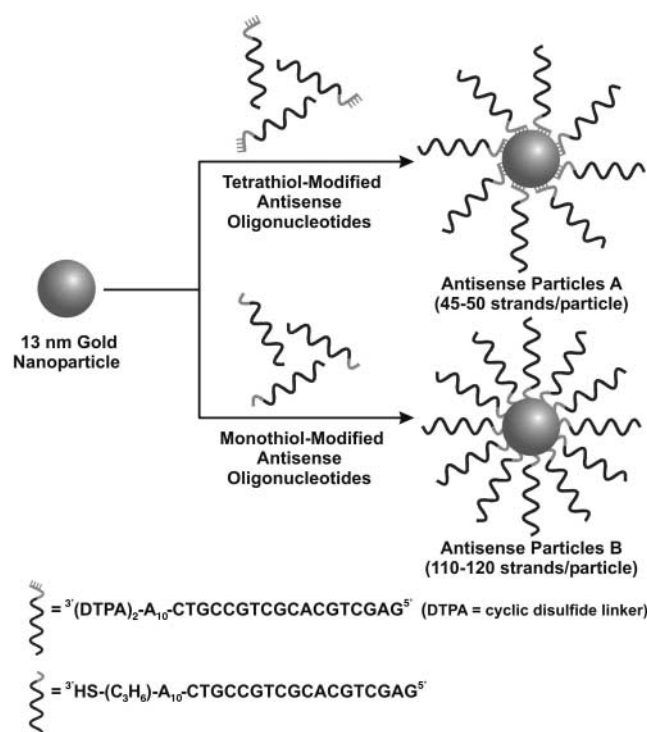


Fig 1. Preparation of antisense Au NPs. Citrate-stabilized gold nanoparticles (13 nm ± 1 nm) were functionalized with ASODNs that were premodified with an A₁₀ tether and either two cyclic disulfides (DTPA) or an alkyl-thiol anchoring group to produce Antisense Particles A and B, respectively.

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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

546 nm and 633 nm light resulted only in fluorescence from the 5' Cy5.5 fluorophore, which could be attributed to some nuclease digestion of the ASODNs and residual fluorescence due to incomplete quenching by the Au NPs (25) (the Cy5.5 is approximately 9 nm from the gold surface). Because only negligible 3' Cy3 fluorescence was observed, we conclude that the ASODNs do, in fact, remain chemically attached to the Au NP surface while inside the cell. In additional experiments, C166 cells were incubated with 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

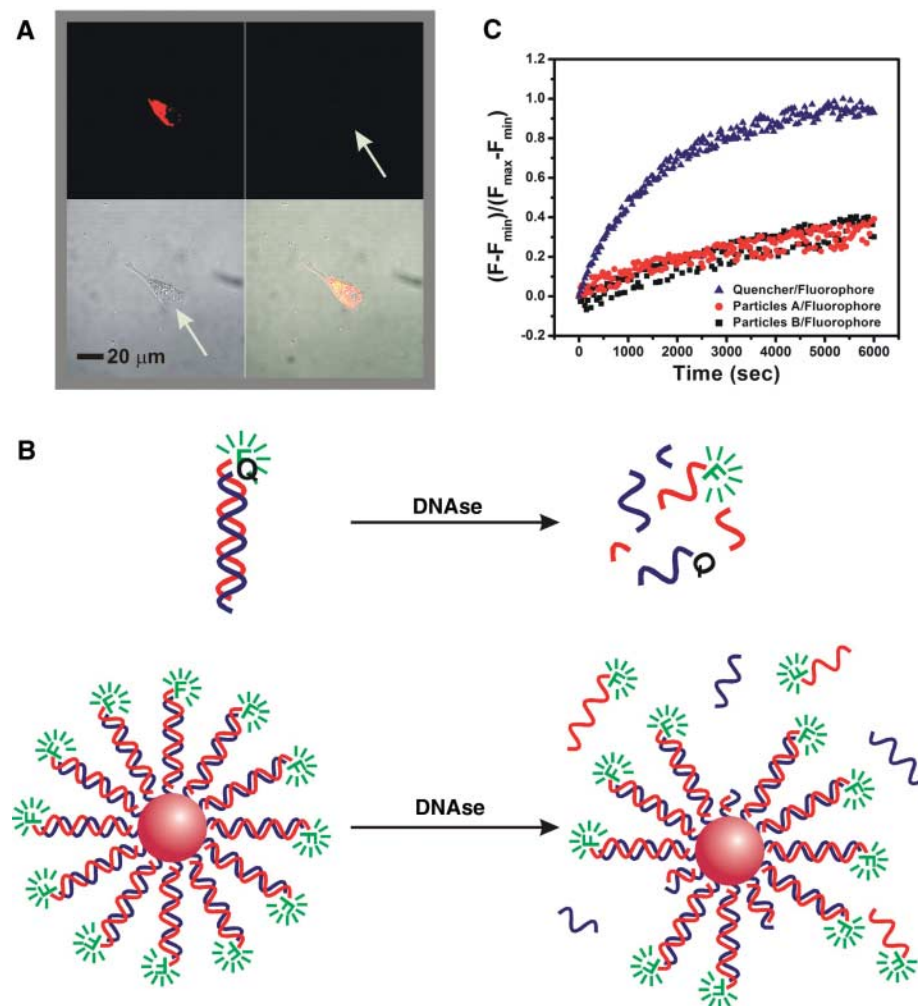


Fig. 2. Experiments aimed at understanding the intracellular stability of antisense nanoparticles. (A) Fluorescent microscopy images of C166-EGFP cells incubated 48 hours with antisense particle B functionalized with dual-fluorophore labeled ASODNs (3' Cy3 and 5' Cy5.5) only reveal fluorescence from 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 $F_{\min}$ is the fluorescence of the mixture at its initial, fully hybridized state and $F_{\max}$ is the maximum fluorescence of the system at 80°C where all of the oligonucleotides are dehybridized.

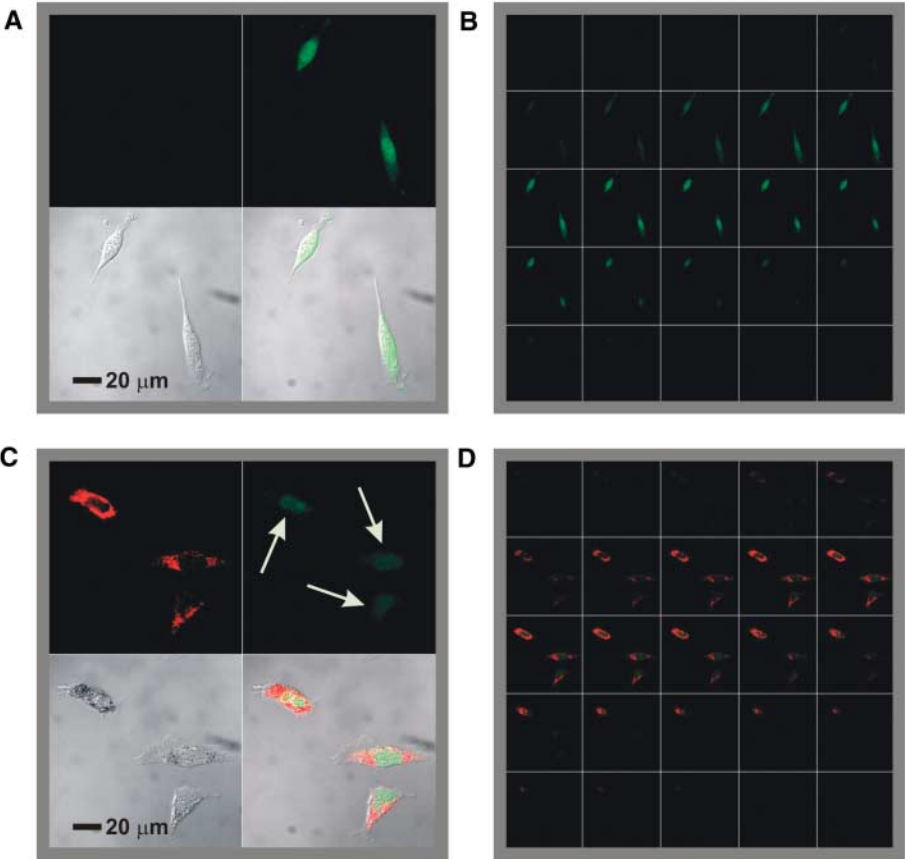


Fig. 3. Confocal fluorescence microscopy images showing EGFP knockdown. (A) Untreated control C166-EGFP cells (upper left, Cy5.5 emission, 706 to 717 nm; upper right, EGFP emission, 500 to 550 nm; lower left, transmission image of cells; lower right, composite overlay of all three channels) showed a significant amount of emission throughout the cell. (B) 1 μ m sectioning images of control cells. (C and D) Cells treated with antisense particles A or B showed a decrease in the amount of EGFP emission.

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 [see (23) 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

Table 1. Performance characteristics of antisense nanoparticles.

Antisense experiment	Observed toxicity	Approximate binding constant	Percent decrease in EGFP expression
Antisense Particles A (0.024 nmol particles 1.08 nmol ASODN)	No	7.1×10^{20}	11 ± 2
Antisense Particles A (0.048 nmol particles 2.16 nmol ASODN)	No	7.1×10^{20}	14 ± 0.4
Antisense Particles B (0.024 nmol particles 2.64 nmol ASODN)	No	2.6×10^{22}	14 ± 1
Antisense Particles B (0.048 nmol particles 5.28 nmol ASODN)	No	2.6×10^{22}	20 ± 4
Nonsense Particles A (0.048 nmol particles)	No	N/A	0 ± 3
Nonsense Particles B (0.048 nmol particles)	No	N/A	0 ± 2
Lipofectamine 2000 (0.024 nmol ASODN)	No	6.7×10^{20}	6 ± 0.2
Lipofectamine 2000 (2.64 nmol ASODN)	Yes	6.7×10^{20}	N/A
Cytofectin (0.024 nmol ASODN)	No	6.7×10^{20}	7 ± 0.7
Cytofectin (2.64 nmol ASODN)	Yes	6.7×10^{20}	N/A

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

amount of ASODNs delivered using antisense nanoparticles (0.024 nmol ASNP: 2.64 nmol ASODN); however, extreme toxicity was observed in both cases as measured by cell death, so we lowered the amount of ASODN and transfection reagent to the point that one strand of transfected ASODN was equivalent to one antisense particle (0.024 nmol ASNP: 0.024 nmol ASODN). In these cases, ASODNs transfected with either Lipofectamine or Cytofectin resulted in only ~6 to 8% knockdown in EGFP expression (Table 1). Evaluation of multiple cell lines (23) to determine toxicity limits of the ODN-Au NPs have not shown any appreciable cell death even with high (0.12 nmol) particle loadings.

Although these systems have not yet been optimized for maximum efficacy, the ability to systematically control the oligonucleotide loading on the NP surface has allowed us to identify several features that make them attractive candidates for antisense studies and therapies. The ability to modify the gold nanoparticle surface allows one to realize unusual cooperative properties that lead to enhanced target binding and allows the introduction of a variety of functional groups that have proven to be informative in terms of studying how the structure works within a cell. Moreover, this platform will allow us and others to add functionality (31, 32) that could direct the oligonucleotide-modified nanoparticle agents to specific cell

types and different components within the cell, thus opening the door for new possibilities in the study of gene function and nanotherapies.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S4

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Preparation of Poly(diiododiacetylene), an Ordered Conjugated Polymer of Carbon and Iodine

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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 diyne 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 single-atom iodine side groups. The monomer, diiodobutadiyne, forms co-crystals with 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 proper-

ties. Recently examined applications include photovoltaic cells, organic light-emitting diodes (OLEDs), field-effect transistors, and chemical sensors (2–5). Polyacetylene, 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 poly-

mers 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. Polydiacetylenes, in which every other double bond in the polyacetylene backbone is replaced by a triple bond, are potent multiphoton absorbers that have applications in optical limiters, waveguides, and thermometric sensors (2).

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 diynes 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 diynes 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

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Supporting Online Material for

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for Intracellular Gene Regulation**

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This PDF file includes:

Materials and Methods
Figs. S1 to S4
References

Supporting Information for:

Oligonucleotide-Modified Gold Nanoparticles for Intracellular Gene Regulation

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Abigail K.R. Lytton-Jean, Min Su Han, and Chad A. Mirkin*

Nanoparticle Synthesis. Citrate-stabilized gold nanoparticles (13 ± 1 nm) were prepared using published procedures.^{si}

DNA Synthesis. Oligonucleotides were synthesized on an Expedite 8909 Nucleotide Synthesis System (ABI) using standard solid-phase phosphoramidite methodology. All bases and reagents, including Cy dyes, sulfurizing reagent (3H-1,2-Benzodithiole-3-one-1,1-dioxide), and dithiolphosphoramidite (DTPA) (1,2-Dithiane-4-O-Dimethoxytrityl-5-[(2-cyanoethyl)-N,N-diisopropyl]-phosphoramidite), were purchased from Glen Research. Sulfurizing Reagent was used to generate phosphorothioate linkages between the two terminal 3' adenines and the two terminal 5' bases (see sequences below). Universal CPG was used for the tetra-thiol oligonucleotides while 3' C₃H₆-thiol CPG (1-O-Dimethoxytrityl-propyl-disulfide,1'-succinyl-lcaa-CPG) was used for the mono-thiol oligonucleotides. All oligonucleotides were purified using published procedures^{si}; however, the cyclic disulfide moieties in the tetra-thiol oligonucleotides were not cleaved with dithiothreitol (DTT). Methods described in Gene Therapy Systems company literature (Cytofectin product insert) were used to further purify the antisense oligonucleotides. After purification, the oligonucleotides were lyophilized and stored at -78 °C until use.

Tetra-thiol Oligonucleotides:

Antisense: 3'(DTPA)₂A-A-A-A-A-A-A-A-A-ACTGCCGTCGCACGTCG-A-G^{5'}
Nonsense: 3'(DTPA)₂A-A-A-A-A-A-A-A-T-T-A-A-C-T-A^{5'}

Monothiol Oligonucleotides:

Antisense: 3'HS(C₃H₆)A-A-A-A-A-A-A-A-A-ACTGCCGTCGCACGTCG-A-G^{5'}
Nonsense: 3'HS(C₃H₆)A-A-A-A-A-A-A-A-T-T-A-A-C-T-A^{5'}

EGFP Antisense Oligonucleotide:

3'C-T-GCCGTCGCACGTCG-A-G^{5'}

Dual-Fluorophore Oligonucleotides:

3'(DTPA)₂Cy3A-A-A-A-A-A-A-A-A-ACTGCCGTCGCACGTCG-A-GCy5.5^{5'}
3'HS(C₃H₆)Cy3A-A-A-A-A-A-A-A-A-ACTGCCGTCGCACGTCG-A-GCy5.5^{5'}

****Note: hyphens indicate phosphorothioate linkage****

Oligonucleotide-Functionalized Particle Preparation. Thiol-modified or cyclic-disulfide-modified oligonucleotides were added to 13 ± 1 nm citrate-stabilized gold nanoparticles (~ 3 nmol oligonucleotide per 1 ml of 10 nM colloid). After 20 min, SDS (10% solution in NANOpure™ water, 18.2 MΩ) was added to bring the mixture to 0.1 % SDS, phosphate buffer (0.1 M; pH = 7.4) was added to bring the mixture to 0.01 M phosphate, and sodium chloride (2.0 M solution in NANOpure™ water) was added to bring the NaCl concentration to 0.1 M. The resulting mixture was shaken gently (30 min.), after which two more additions of 2.0 M NaCl were added in thirty minute intervals to bring the final mixture to 0.3 M NaCl. This final mixture was gently shaken (24 h) to complete the oligonucleotide functionalization process. The particles were centrifuged (13000 rpm, 20 min.; 3X) and resuspended in 1X PBS (Hyclone).

Cell Culture and Particle Incubation. C166, C166-GFP^{sii}, NIH 3T3, RAW264.7, and HeLa cells were obtained from American Tissue Culture Collection (ATCC) and were grown in 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS. Cells were seeded at a density of 15,000 cells/cm² and were grown for 24 hours prior to treatment with particles. After 24 hours, the cells were washed with 1X PBS and fresh media was added. The particles (0.024 nmol or 0.048 nmol) were filtered (0.20 μm acetate syringe filter) and added to the media of the freshly washed cells. After 48 hours, the cells were washed in 1X phosphate-buffered saline (PBS) (Hyclone), trypsinized (Trypsin-EDTA), and collected for analysis. Cells were analyzed by a trypan-blue dye (Sigma-Aldrich) to determine percent viability.

Fluorescence Determination of EGFP Expression. Fluorescence was measured using a SpectraMax Gemini EM Microplate Spectrofluorometer (Molecular Devices). Cell samples were lysed in 1X Cell Lysis Buffer (20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 1 mM Na₂EDTA; 1 mM EGTA; 1% Triton 2.5 mM) (Cell Signaling Technologies). Extracted protein samples were placed in 96 well black/clear bottom plates (Costar) and excited at a wavelength of 488 nm. Fluorescence emission was measured by taking the area under the curve from 524 to 550 nm. All samples were measured in triplicate.

To determine percent knockdown of EGFP, a calibration curve was first generated for each set of samples using lysates of untreated C166-EGFP cells of varying concentrations. Following fluorescence measurements, a BCA assay (Pierce) was performed to determine the protein concentrations of each sample. From these data, a standard curve of fluorescence versus protein concentration was generated. Concurrent with the measurement of the standard fluorescence curve, the fluorescence of the experimental samples were collected (termed “actual” fluorescence). A BCA assay was used to determine the bulk protein concentration of these samples. The protein concentration values were used to extrapolate from the standard calibration curve a “predicted” fluorescence value for each sample. The “predicted” value was compared to the “actual” fluorescence emitted by the sample. The ratio of “actual” fluorescence to “predicted” fluorescence for each sample was normalized to the ratios for the untreated controls to determine percent knockdown.

Imaging. Cells were grown on a glass coverslip which was placed at the bottom of a 6 well tissue culture plate (Corning). Oligonucleotide-functionalized particles (0.012 nmol) were then added to the wells. Following incubation (48 hours), the cells were washed and mounted on glass slides for imaging. All imaging was performed using two-photon excitation (TPE) laser scanning microscopy on a Zeiss 510 LSM (upright configuration) equipped with a 63x oil-immersion objective (Carl Zeiss, Inc.). In all experiments, the pinhole and gain settings of each individual collection channel were determined using untreated control cells. The settings were held constant throughout the experiment. Sectioning images were obtained at 1 μ m intervals.

EGFP Knockdown Studies. Cellular EGFP fluorescence from C166-EGFP cells was excited using an argon laser source at 488 nm and emission was collected between 500 and 550 nm. Cy5.5 (which was used to label the 5' ends of the ASODNs on the gold nanoparticle) was excited with a HeNe laser source (633 nm), and its emission was collected at a different channel between 706 and 717 nm.

Dual-Fluorophore Particle Stability Studies. Cellular EGFP fluorescence from C166-EGFP cells was not collected in this experiment. Cy5.5 (which was used to label the 5' ends of the ASODNs on the gold nanoparticle) was excited with a HeNe laser source (633 nm) and its emission was collected (706 to 717 nm). Cy3 (which was used to label the 3' ends of the ASODNs on the gold nanoparticle) was excited using a second HeNe laser source (543 nm), and its emission was collected from a separate channel between 565 and 615 nm.

Cellular Particle Uptake Studies. For C166-EGFP cells, NIH-3T3 cells, and HeLa cells, Cy5.5 was used to label the ASODNs on the antisense particles, and therefore identical conditions as described above for Cy5.5 were used. In the case of RAW 264.7 and MDCK cells, Cy3 was arbitrarily chosen to label the particle-bound ASODNs. For these experiments, Cy3 was excited using a HeNe laser source (543 nm) and its emission was collected between 556 and 589 nm.

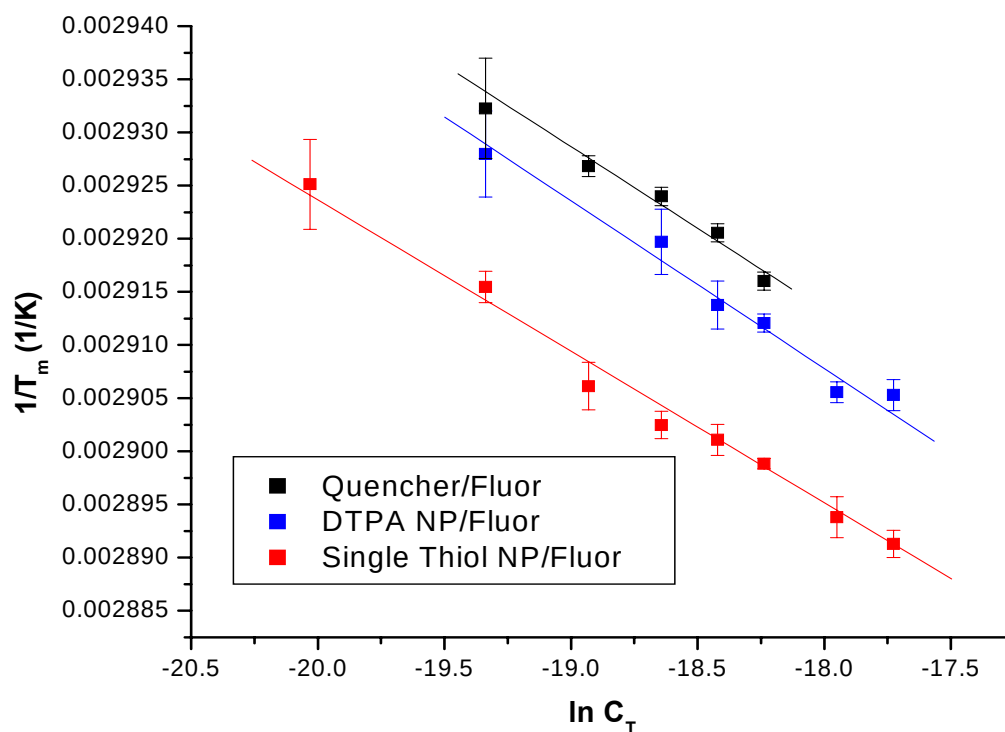
Binding Constant Studies. All binding constant measurements were performed according to published procedures.^{siii} In these studies, fluorescein was chosen as the molecular fluorophore and Dabcyl was used as the molecular quencher.

Glutathione Resistance Studies. Glutathione (GSH) was purchased from Sigma-Aldrich. Concentrations of GSH ranging from 500 nM to 10 mM were added to dual-fluorophore oligonucleotide-modified gold nanoparticles prepared as described above. Samples were incubated at 37°C for up to 48 h after GSH addition. At time points of 0, 1, 8, 24, and 48 h, 1 mL aliquots of the samples were centrifuged (13000 rpm, 20 min) to remove particles. The fluorescence of the supernatant was measured between 695 and 800 nm by excitation with 688 nm light and the area under the curve between 730 and 800 nm was measured and compared to a standard curve. Percentage of release by GSH

was determined by normalizing the data to dithiothreitol (DTT) mediated release (0.1 M final concentration DTT causes 100% release of oligonucleotides from the particles).^{siv}

Nuclease Resistance Studies. DNase I was purchased from New England Biolabs Inc. Dabcyl- tagged antisense DNA (having two terminal phosphorothioate linkages on both ends) was synthesized ‘in-house’ and Alexa Fluor®-tagged complementary DNA was purchased from Integrated DNA Technologies. All stock solutions were prepared by dissolving ultrapure BSA (Ambion) (0.05 mg/mL final concentration), Mg^{2+} (0.25 mM final concentration), in 0.15 M PBS buffer (pH 7.0) (Hyclone). The following fluorometer settings were used: excitation wavelength, 495 nm; emission wavelength, 520 nm; slit width (all slits), 3 nm; 1 measurement every 30 seconds; assay period, 6000s.

All substrate stock solutions were incubated 6 h before use and compositions of the substrate stock solutions were as follows: control (Dabcyl-tagged DNA, 900 nM; Alexa Fluor® tagged DNA, 900 nM), Particles A (nanoparticle, 20 nM; Alexa Fluor® tagged DNA, 900 nM), Particles B (nanoparticle, 10 nM; Alexa fluor® tagged DNA, 900 nM). Reaction mixtures (1 mL) consisted of 50 units DNase I and 10 times diluted substrate stock solution. The change in the fluorescence at 520 nm was measured immediately at room temperature. F_{max} was the fluorescence of the reaction mixture (melted DNA) at 80 °C, and F_{min} was the fluorescence of the reaction mixture at the initial, fully hybridized state.



	ΔH (kcal/mol)	ΔS (kcal/mol*K)	ΔG (kcal/mol)	K at 298K
Quencher-Fluor	-129 ± 14.4	-0.339 ± 0.038	-28.4 ± 14.4	6.7×10^{20}
TetraThiol NP/Fluor	-126 ± 15.7	-0.327 ± 0.041	-28.4 ± 15.7	7.1×10^{20}
MonoThiol NP/Fluor	-139 ± 9.5	-0.365 ± 0.025	-30.6 ± 9.5	2.6×10^{22}

Fig. S1. The melting temperatures of molecular-quencher-modified ASODNs and ASNP B with complementary fluorophore-labeled target oligonucleotides were measured at various concentrations by monitoring the increase in fluorescence with temperature. Thermodynamic analysis of these data reveal that ASNP Particle B has an equilibrium binding constant more than 35 times larger than unmodified ASODNs. Identical experiments were performed using Particles A; it was found that the binding constant for these particles was approximately equal to that of unmodified ASODNs.

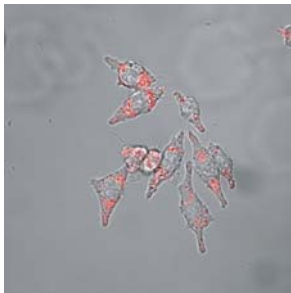
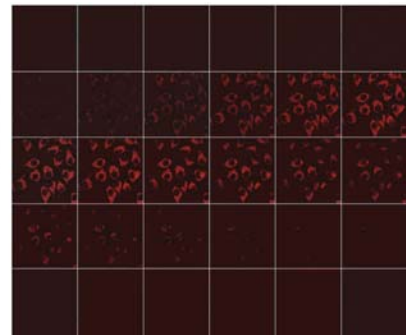
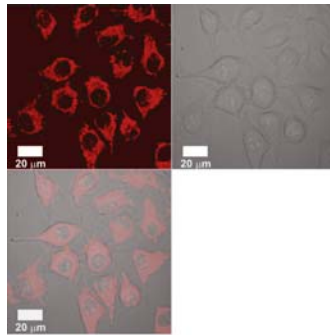
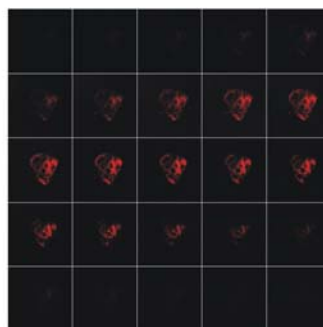
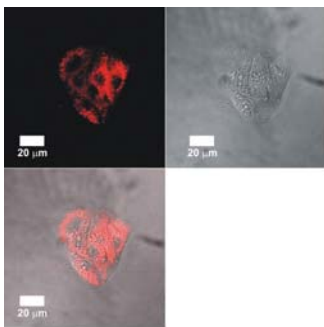
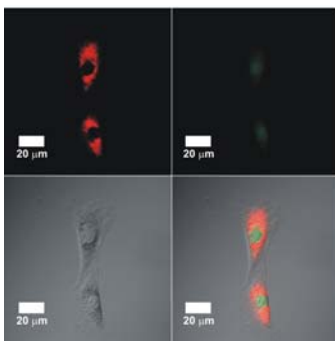
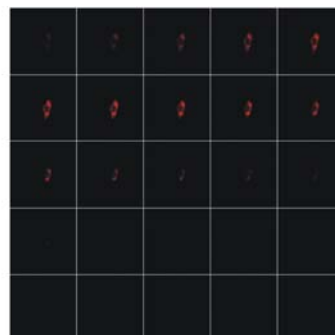
A**B****C****D****E**

Fig. S2. Transfections of different cell types with oligonucleotides nanoparticles. **(A)** RAW 264.7 transfected with Cy3 labeled ASNPs. **(B)** HeLa. Left: (clockwise from upper left) Cy5.5 channel, transmission channel, overlay channel. Right: Sectioning images. **(C)** MDCK. Left: (clockwise from upper left) Cy5.5 channel, transmission channel, overlay channel. Right: Sectioning images. **(D)** NIH3T3 EGFP. Left: (clockwise from upper left) Cy5.5 channel, EGFP channel, overlay channel, transmission channel. Right: Sectioning images. Note that there is also knockdown observed in this EGFP expressing line. **(E)** C166 EGFP. Left: (clockwise from upper left) Cy5.5 channel, EGFP channel, overlay channel, transmission channel. Right: Sectioning images.

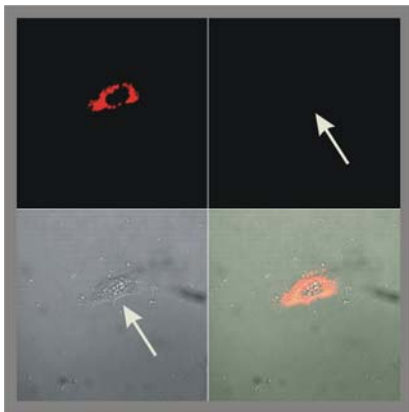


Fig. S3. Fluorescent microscopy images of C166-EGFP cells incubated (48 hours) with ASNP A functionalized with fluorophore labeled ASODNs (3' Cy3 and 5' Cy5.5) only reveal fluorescence from Cy5.5 (706 to 717 nm) (**A**, upper left). Negligible fluorescence is observed in the emission range of Cy3 (565 to 615 nm) (**A**, 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.

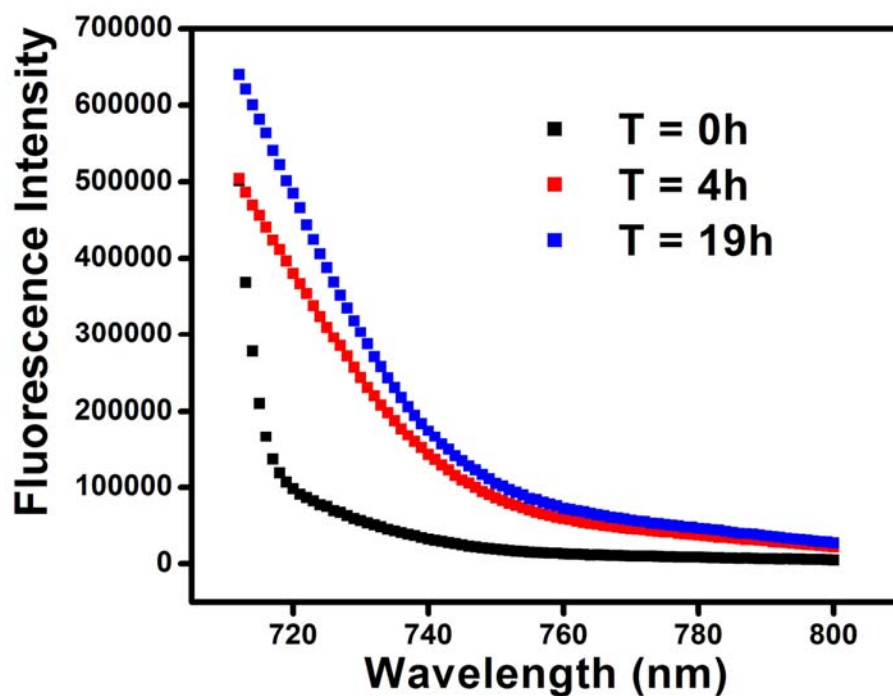


Fig. S4. Fluorescence analysis of lysate before (T = 0 h) and after treatment with DTT (T = 4 h, 19 h).

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^{sii} S.J. Wang, *In Vitro Cell. Dev. Biol. Anim.* **32**, 292 (1996).

^{siii} A.K.R. Lytton-Jean, C.A. Mirkin, *J. Am. Chem. Soc.* **127**, 12754 (2005).

^{siv} Demers et al., *Anal. Chem.* **72**, 5535 (2000)