

## Gene Regulation with Polyvalent siRNA–Nanoparticle Conjugates

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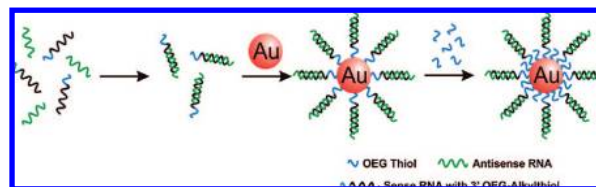
Over the past decade, researchers have designed, synthesized, studied, and applied polyvalent DNA-functionalized gold nanoparticles (DNA–Au NPs).<sup>1</sup> These efforts have resulted in a new fundamental understanding of hybrid nanostructures,<sup>2</sup> important and in certain cases commercially viable detection and diagnostic assays,<sup>3</sup> and the ability to program materials assembly through the use of DNA synthons.<sup>1,4</sup> Polyvalent DNA–Au NPs have several unique properties, such as sharp and elevated melting temperatures,<sup>2b</sup> enhanced binding properties<sup>2c</sup> (as compared with free strands of the same sequence), and distance-dependent optical properties.<sup>5</sup> In agreement with research on polyvalent molecular systems,<sup>6</sup> the high surface DNA density and the ability of the nanoparticles to engage in multidentate interactions are the proposed origin of these unique properties.

Recently, we demonstrated the utility of the polyvalent DNA–Au NP for antisense gene regulation, where the unique ensemble properties of the conjugate confer several important advantages in the context of intracellular target recognition and binding.<sup>7</sup> These properties include resistance to nuclease degradation and high cellular uptake as a result of their oligonucleotide functionalization. Although antisense DNA is an important way of regulating genes, an even more promising route is through the use of siRNA.<sup>8</sup> However, no methods have been developed for utilizing polyvalent particles and their unusual properties to load and transport RNA across cell membranes. Indeed, one must develop synthetic routes and materials that overcome one of the most challenging problems associated with RNA, most notably its chemical instability.

Based upon our observations with DNA-modified particles, we hypothesized that gold nanoparticles densely functionalized with synthetic RNA oligonucleotides would take advantage of the ensemble properties that result from the dense surface functionalization of oligonucleotides, increase the stability and efficacy of the bound RNA, while retaining the ability to act in the highly potent and catalytic RNA interference pathway. While others have prepared siRNAs with chemical modifications, peptide conjugation, or as nanomaterials coated or embedded with small or synthetic RNA for the purposes of enzymatic detection, siRNA delivery, or even control of nanoparticle shape,<sup>9</sup> here, we describe a unique nanoparticle conjugate that has a dense, covalently attached RNA monolayer. This functionalization results in a conjugate with unique properties that make it a potent material for cellular uptake, serum stability, and genetic regulation without the use of auxiliary transfection strategies or chemical modifications.

The synthesis of polyvalent RNA–nanoparticle conjugates (RNA–Au NPs) requires that all components are free of nucleases, such as RNase, which degrades RNA ligands and results in unstable Au NP interactions by exposing the Au surface (as evidenced by aggregation). While conditions for preparing RNase-free organic components and solutions are well established, conditions for creating RNase-free inorganic gold nanoparticles have not yet been described. We determined that treatment of citrate-capped Au NPs with diethylpyrocarbonate (DEPC) followed by autoclaving at 121 °C for 60 min yields sterile, RNase free gold colloids, as measured using an RNase activity kit (Supporting

**Scheme 1.** Preparation of Polyvalent RNA Gold Nanoparticle Conjugates<sup>a</sup>



<sup>a</sup> RNA duplexes (containing a double-stranded RNA sequence, an ethylene glycol spacer, and an alkylthiol group) are formed via hybridization and subsequently incubated with RNase free nanoparticles. Further passivation with oligoethylene glycol-thiol (OEG-Thiol) adds additional stability in cell culture.

Information, SI). Importantly and quite surprisingly, the optical and physical properties of the nanoparticles are unaffected by this relatively extreme treatment, as measured by UV spectroscopy and TEM analysis (SI). The resultant RNase-free nanoparticles were amenable to further modification by thiolated oligonucleotides using published procedures.<sup>2a</sup> Without this pretreatment, subsequent functionalization with RNA could not be accomplished, presumably due to rapid degradation of the RNA-based surface capping ligands. Duplexes composed of a 27-base RNA strand, and 25-base complement terminated with an ethylene glycol spacer and alkylthiol, were hybridized and added to the RNase-free Au NPs, where they were allowed to chemisorb via the thiol–gold bond. For this work, the sequences were designed to target the firefly luciferase gene (SI).

To prevent dehybridization of the RNA duplex, the salt concentration of the Au NP solution was adjusted to 0.1 M with NaCl prior to duplex addition. Subsequently, the salt concentration was increased from 0.1 to 0.3 M NaCl over 12 h with brief ultrasonication following each addition to increase the coverage of oligonucleotides on the nanoparticle surface.<sup>10</sup> To yield a more stable conjugate, RNA-functionalized particles were treated with 30  $\mu\text{mol/mL}$  of oligoethylene glycol-thiol (OEG-Thiol) as an additional surface passivating ligand (Scheme 1). OEG-Thiol passivation was found to stabilize these nanomaterials under cell culture conditions for extended periods of time (SI).

Following synthesis, RNA–Au NPs were purified by three successive centrifugation, supernatant removal, and addition of phosphate buffered saline (PBS) steps at 4 °C. Refrigerated centrifugation was necessary to prevent duplex dehybridization due to heating caused by the centrifugation process. To determine dsRNA loading, antisense strands were labeled with Cyanine 3 (Cy3) fluorescent dye. The number of duplexes per particle was calculated by oxidizing the gold using 25  $\mu\text{M}$  potassium cyanide, measuring the number of fluorescent antisense strands (indicative of formed duplexes), and dividing by the concentration of nanoparticles. Each RNA–gold nanoparticle conjugate contains  $33 \pm 4$  RNA duplexes per 13 nm Au NP.

The ability of the conjugates to enter cells was investigated by confocal microscopy using fluorescent (Cyanine 5) conjugates prepared as above. RNA–Au NPs were added to cultures of HeLa cells. Imaging

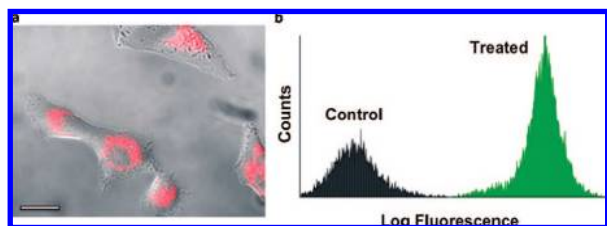


**Figure 1.** Cellular uptake of RNA–Au NPs. (a) Fluorescence microscopy images of HeLa cells incubated for 6 h with RNA–Au NPs (Cy5 labeled RNA). Scale bar is 20  $\mu\text{m}$ . (b) Flow cytometry analysis comparing RNA–Au NP treated cells to untreated controls.

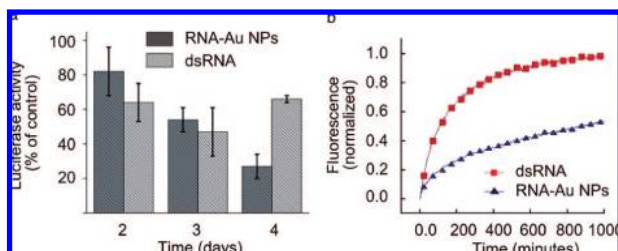


**Figure 2.** (a) Knockdown of luciferase expression over 4 days. (b) Stability of RNA–Au NPs. Comparison of the stability of dsRNA (red) and RNA–Au NPs (blue) in 10% serum.

studies reveal fluorescence throughout the cytoplasm of HeLa cells after 6 h (Figure 1a). It is interesting to note that, like DNA–Au NPs, RNA–Au NPs require no transfection agent to enter cells.<sup>11</sup> Indeed, analytical flow cytometry confirmed uptake of the RNA–Au NPs in >99% of the cell population (Figure 1b).

Having determined that RNA–Au NPs are internalized by cells, we next examined the intracellular activity of RNA–gold nanoparticle conjugates. We conducted protein knockdown studies in HeLa cells using a transfected luciferase plasmid as a target for this model system. Briefly, HeLa cells were seeded in 96 well plates and transfected with a plasmid containing both Renilla and firefly luciferase. The media was replaced 24 h after plasmid introduction with media containing the functionalized RNA–Au NPs (3 nM nanoparticle concentration,  $\sim 100$  nM RNA duplex concentration) directed against firefly luciferase. For comparison, we transfected the same number of luciferase siRNA duplexes (100 nM) using the commercial agent Lipofectamine 2000. Quantification of luciferase expression reveals that the nanoparticle agents down-regulate firefly luciferase in a dose and time dependent manner. Control (Renilla) expression is not affected by RNA–Au NPs designed against firefly luciferase, indicating that the knockdown is also sequence specific. No changes in cell viability that would indicate off-target effects were observed, although this remains an active area of investigation. Interestingly, the results of three independent experiments with the RNA–Au NPs show knockdown that exceeds that of the free RNA 4 days after treatment ( $73 \pm 7\%$  RNA–Au NPs vs  $33 \pm 2\%$  free, Figure 2a).

We hypothesize that the persistent knockdown of luciferase may be the result of the stabilization of the RNA on the nanoparticle. In stability experiments, conjugates incubated in serum show greatly enhanced stability relative to their molecular RNA counterparts. For example, in the presence of 10% serum, RNA–Au NP conjugates have a 6 times greater half-life than molecular RNA duplexes ( $816 \pm 59$  min vs  $133 \pm 30$  min, Figure 2b). These data indicate that nanoparticle conjugation provides significant protection from degradation in an extracellular context. This stabilization is likely due to the high local salt concentration, which is known to deactivate enzymatic activity.<sup>12</sup> Since the extracellular lifetime of RNA is of great importance for their storage, handling, and potential therapeutic application,

nanoparticle conjugation may provide a significant advantage for the protection and delivery of functional RNA ligands. Importantly, this enhanced stability does not require chemical modifications to protect the integrity of the RNA.

We have developed the first polyvalent RNA–gold nanoparticle conjugates, which can be used to effectively regulate genes in the context of RNA interference. In contrast to other materials for introducing RNA to a cell, these conjugates are not simply a delivery tool for RNA, but rather single entity agents which take advantage of the cooperative properties that result from the arrangement and high density of the surface ligands.

The novel conjugate described here is a densely functionalized nanomaterial that is protected from degradation in a manner that enhances knockdown compared to conventional lipid carriers and enters cells without transfection agents. Note that in recent work we have shown that DNA density results in a decrease in nuclease activity as a result of high local salt concentration.<sup>13</sup> The enhanced stability we observe here for the RNA–Au NPs may be due to the same phenomena. Given their straightforward synthesis, these RNA–Au NPs, in addition to useful intracellular gene regulation materials, may be promising candidates for therapeutic and diagnostic applications.

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**Supporting Information Available:** Experimental conditions, sequences, and materials synthesized. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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(Supporting Information)

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**RNA synthesis.** RNA oligonucleotides were synthesized using TOM- RNA reagents (Glen Research) and a MerMade 6 (Bioautomation) or commercially made (Integrated DNA Technologies). The oligonucleotides synthesized in-house were purified using TOP-RNA cartridges (Varian). The sequences used for this study were: luciferase sense, 5' - Phosphate rCrGrA rCrUrU rCrGrU rGrCrC rArGrA rGrUrC rUrUrU rCrGAC Spacer 18 Spacer 18-Thiol - 3', luciferase antisense, 5'- rGrUrC rGrArA rArGrA rCrUrC rUrGrG rCrArC rGrArA rGrUrC rGrUrA -3', Cy3 luciferase, 5'-Cy3 rGrUrC rGrArA rArGrA rCrUrC rUrGrG rCrArC rGrArA rGrUrC rGrUrA -3', Cy5 luciferase, 5'-Cy5 rGrUrC rGrArA rArGrA rCrUrC rUrGrG rCrArC rGrArA rGrUrC rGrUrA -3', luciferase dabcyl, 5'-rCrGrA rCrUrU rCrGrU rGrCrC rArGrA rGrUrC rUrUrU rCrGA C-dabcyl -3', Renilla luciferase sense, 5'-Phosphate rGrGrA rGrGrA rCrGrC rUrCrC rArGrAr UrGrA rArArU rGrGGT Spacer 18 Spacer 18-Thiol-3', Renilla luciferase antisense, 5' rArCrC rCrArU rUrUrC rArUrC rUrGrG rArGrC rGrUrC rCrUrG-3'.

**Gold particle synthesis.** Citrate-stabilized gold nanoparticles (13 nm) were prepared using published procedures.<sup>1</sup> Following synthesis, particles were treated with 0.1% diethylpyrocarbonate (DEPC) for 12 hours with stirring, then autoclaved at 121°C for 60 minutes. This preparation caused no differences in particle morphology as determined by UV spectroscopy or Transmission Electron Microscopy (TEM) analysis (Figure S1). Subsequent ligand functionalization also was not affected by this treatment. Testing of RNase activity in these solutions using an RNaseAlert kit (Ambion) showed no detectable RNase activity as compared to controls or untreated particles (Figure S2).

**siRNA nanoparticle preparation and characterization.** Pre-formed, thiolated RNA duplexes (1000 nM) were incubated with RNase-free solution of Au NPs (10 nM) which had been adjusted with 0.1 M NaCl. The mixture was aged in solutions of increasing salt concentration (from 0.1 to 0.3 M NaCl) and sonicated to increase the coverage of oligoribonucleotides on the surface. Oligoethylene glycol was added (30 µmol/mL final concentration) 24 hrs after duplex addition. Addition at this concentration did not change duplex loading. After functionalization, the particles were purified by centrifugation at (13,000 rpm, 20 mins) at 4°C, and re-suspended in sterile phosphate buffered saline (PBS 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4). This process was repeated 3 times. The number of RNA duplexes per gold nanoparticle was determined by using a fluorophore-labeled (Cy3) antisense strand. Briefly, the conjugate was treated with 25 µM potassium cyanide to oxidize the gold nanoparticles. Fluorescence was measured using a Jobin Yvon Fluorolog FL3-22 with excitation at 550 nm and measuring emission from

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<sup>1</sup> Frens, G., Controlled Nucleation for the Regulation of Particle-Size in Monodisperse Gold Suspensions. *Nature Physical Science* **1973**, 241, 20-22.

560 to 620 nm in 1 nm increments and compared to a standard curve. The number of duplexes per particle was determined as  $33 \pm 4$  per 13 nm gold nanoparticle.

**Cell preparation.** HeLa cells (ATCC) were grown in Eagle's minimal essential medium (EMEM), with 10 % heat inactivated fetal bovine serum and maintained at 37 °C in 5 % CO<sub>2</sub>. Cells were seeded in 96 well plates and grown for 1 day prior to transfection of a plasmid (psiCHECK 2, Promega) which contains both firefly luciferase and Renilla genes. Plasmid (0.2 µg per well) was added using Lipofectamine 2000 (Invitrogen) according to the the manufacturer's recommendations. After plasmid introduction (24 hrs) the media was replaced with reduced serum media containing the functionalized RNA-Au NPs. On day one of treatment, the cells were approximately 70% confluent. At the conclusion of experiments, replicate wells of treated cells were counted and measured for viability using a Guava EasyCyte Mini (Guava Technologies). Viability after incubation was > 98% for cells treated with RNA-Au NPs.

**Gene knockdown.** siRNA nanoparticles (3 nM) were added to luciferase/Renilla cells in 96 well plates at 3 nM concentration. For comparison, cells were treated with 100 nM luciferase siRNA using Lipofectamine 2000 according to the manufacturer's recommended protocol. After 24 hours of treatment, the media was replaced with fresh EMEM. Cells were assayed for luciferase expression using a Dual-Glo (Promega) assay according to the manufacturer's protocols after the indicated number of days. Quantification of luciferase expression was normalized to controls that had not been transfected. In addition, as Renilla expression was not affected, it served as an internal control for normalizing luciferase expression.

**Imaging.** Cells were grown on glass coverslips and treated with nanoparticles functionalized with fluorophore-labeled RNA duplexes. After 6 hours of treatment, the coverslips were removed, washed with PBS, and fixed to a chamber filled with PBS mounted on a glass slide. All images were obtained by scanning confocal microscopy (Zeiss 510 LSM) at 63x magnification and a 633 nm HeNe laser excitation source.

**Flow cytometry.** Cells were treated with fluorescently-labeled (Cy5) siRNA- nanoparticle conjugates. 6 hrs post transfection, cells were trypsinized to remove them from cell culture wells. Flow cytometry was performed using a DakoCytomation CyAn with excitation at 635 nm.

**Nuclease assay.** Cy3-labeled siRNA particles were diluted to a concentration of 5 nM in 90 µL of PBS in a 96-well plate. For the dabcy1-labeled molecular siRNA, the concentration was 150 nM. The microplate was placed into a Photon Technology International FluoDia T70 fluorescence plate reader that was maintained at 37°C. After allowing the sample to equilibrate (10 minutes), 10 µL of fetal bovine serum (FBS, Gemini Bioproducts) was added to bring the samples to 10% serum concentration. To prevent evaporation, the reaction was covered with 40 µL of mineral oil. The fluorescence of the sample (excitation = 530 nm, emission = 570 nm) was measured every 5 minutes for 48 hours. The base line fluorescence was determined from a sample treated with a 10 µL aliquot of PBS in place of FBS. The endpoint of the reaction was determined when no further increase in fluorescence as a function of time was observed. All samples were measured in triplicate.

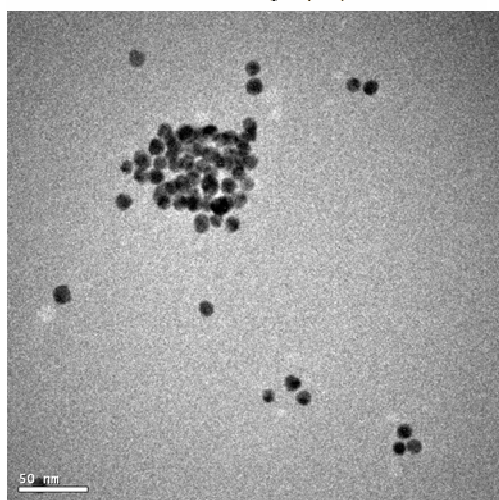
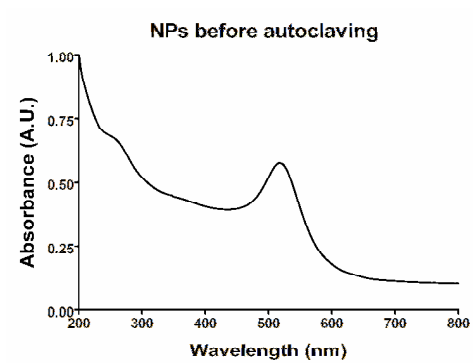
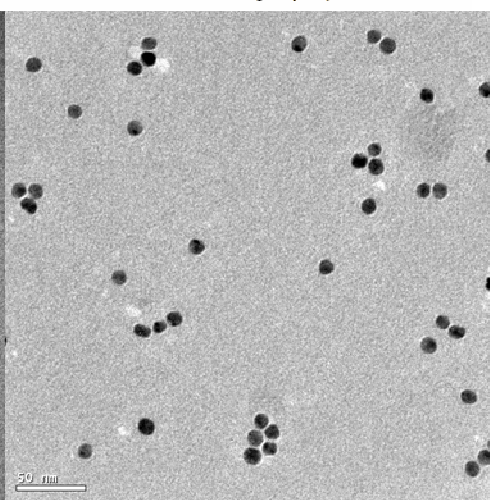
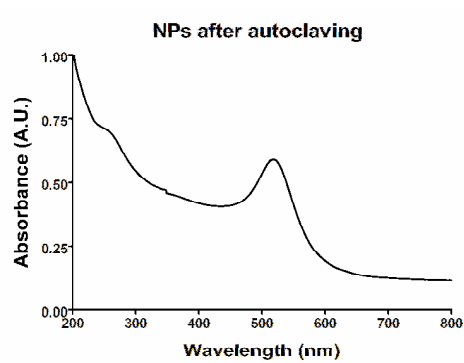
**A****B**

**Figure S1.** Characterization of gold nanoparticles (NPs). Nanoparticle absorbance spectra and TEM imaging before (a) and after (b) autoclaving. Scale bar is 50 nm.

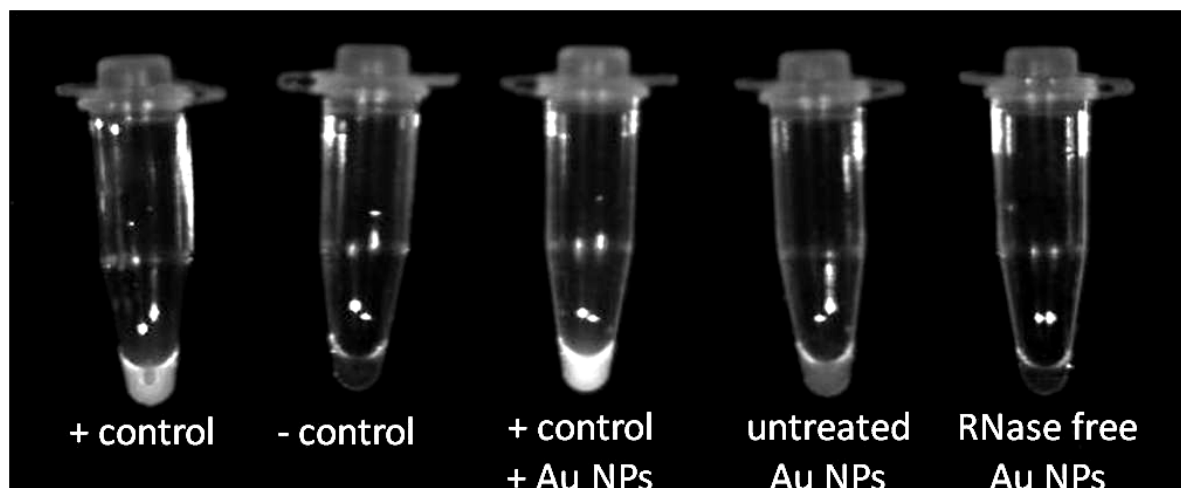


**Figure S2.** Presence of RNases in solutions of gold nanoparticles. Untreated Au NPs show a positive signal, while particles treated to eliminate RNase activity are rendered RNase free.

**A**

**B**

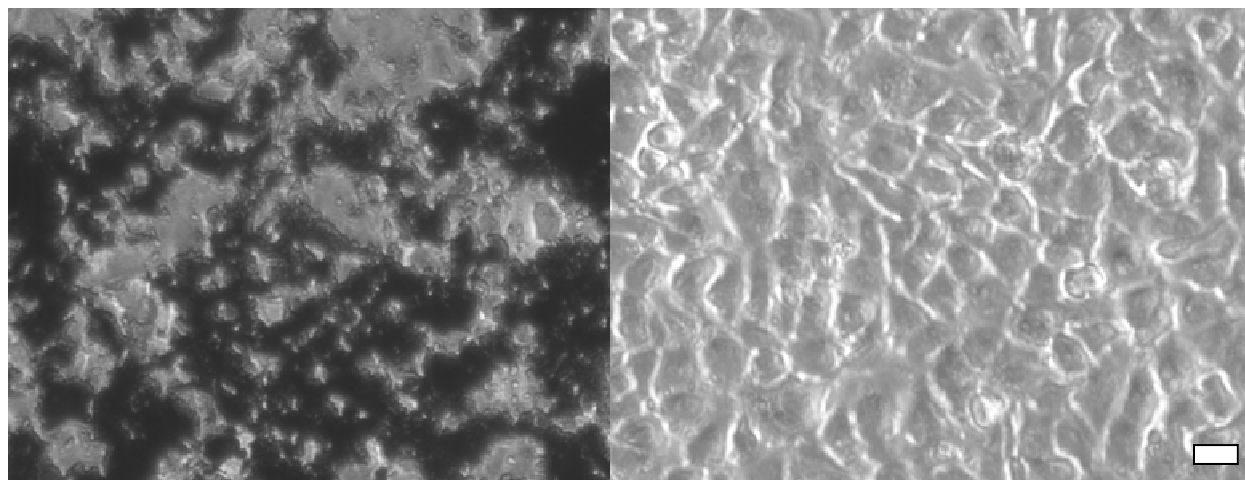


**Figure S3.** Light microscopy images of confluent HeLa cells. In the synthesis of RNA nanoparticle conjugates, 30 $\mu$ mol/mL of oligoethylene glycol-thiol (OEG-thiol) was added as a surface passivating ligand following RNA duplex addition. This addition was found to prevent particle precipitation in culture. (a) siRNA-nanoparticle conjugates in cell culture without OEG-thiol addition show particle precipitation (black). (b) siRNA-nanoparticle conjugates in cell culture with OEG-thiol. Scale bar is 30  $\mu$ m.