Antisense Silver Nanoparticles for Photo-activated Gene Silencing

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ANTISENSE SILVER NANOPARTICLES
FOR PHOTO-ACTIVATED GENE SILENCING

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Master of Science in Biological and Agricultural Engineering

In

The Department of Biological and Agricultural Engineering

by
Paige K Brown
B.S., Louisiana State University, May 2008
May, 2010
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ag</td>
<td>Silver</td>
</tr>
<tr>
<td>Au</td>
<td>Gold</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy-ribonucleic acid</td>
</tr>
<tr>
<td>GNP</td>
<td>Gold Nanoparticle</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intracellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>LSPR</td>
<td>Localized Surface Plasmon Resonance</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA (ribonucleic acid)</td>
</tr>
<tr>
<td>nm</td>
<td>nano-meters</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
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<tr>
<td>Oligo</td>
<td>Oligonucleotide</td>
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<tr>
<td>PC</td>
<td>Photocleavable</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene) glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>Pt</td>
<td>Platinum</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEF</td>
<td>Surface Enhanced Fluorescence</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface Enhanced Raman Scattering</td>
</tr>
<tr>
<td>SNP</td>
<td>Silver Nanoparticle</td>
</tr>
<tr>
<td>ssDNA/dsDNA</td>
<td>single-stranded DNA/double-stranded DNA</td>
</tr>
<tr>
<td>TEM/SEM</td>
<td>Transmission Electron Microscopy/Scanning Electron Microscopy</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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Abstract

The unique properties of noble metal nanoparticles, which include tunable electronic and photonic characteristics, contribute to their potential as novel delivery vectors with enhanced drug stability, cell uptake, and photo-activated functionalities. Silver, as one of best surface-enhancing substrates available for bulk nanostructure synthesis, is a prime choice for investigations of metal nanohybrids as antisense therapy vehicles with special surface plasmon resonance (SPR) enabled functional attributes. The singular photonic properties of silver nanoparticles (SNPs) may contribute to ease of delivery confirmation and in situ photo-activation of protected cargo packed on particle surfaces. Here we show the synthesis and characterization of 40-80nm SNPs designed for enhanced antisense oligonucleotide delivery and photo-activated gene silencing. Non-active (caged) SNP-bound DNA oligonucleotides possess an internal nitrobenzyl photocleavable linker which once exposed to light, initiates disengagement of functional antisense oligonucleotides from the nanohybrid surface. We demonstrate light-triggered, spatiotemporally controlled gene silencing based on SNP-antisense conjugates, which prove to be promising alternative platforms for gene therapy, gene expression studies, and other nanomedicine applications.
Chapter 1. Background and Significance

1.1 Objectives

The objectives of the current thesis project are as follows:

1. Synthesize and characterize 50-60nm diameter citrate-stabilized silver nanoparticles (SNPs)
   a. Evaluate SNP morphology, yield, and optical properties

2. Functionalize SNPs with thiol-modified antisense oligonucleotides possessing internal photo-cleavable (nitrobenzyl) linkers
   a. Evaluate size, morphology, and yield of functionalized SNP-oligo conjugates
   b. Quantify oligonucleotide attachment to SNP-antisense conjugates

3. Evaluate o-nitrobenzyl linker photochemistry on silver nanoparticle surfaces

4. Evaluate the intracellular delivery of SNP-antisense conjugates and photo-induced release of antisense oligonucleotides within an adherent epithelial cell line
   a. Evaluation of antisense functionality against intracellular adhesion molecule-1 (ICAM1) of SNP-antisense photo-activated conjugates within HeLa cells

1.2 Noble Metal Nanoparticles: Properties and Applications

The use of nanostructures, notably colloidal nanoparticles, in molecular diagnostics and drug delivery has emerged and increased significantly within the last decade[1]. Nanostructures are amenable to molecular detection, intracellular diagnostics and therapeutic applications due to their sub-microscopic dimensions (<1 um) and the often unique properties which emerge at these dimensions, and which differ from those of the bulk materials. Due to corresponding size-scales, nanostructures easily interface with biological molecules, such as nucleic acids and proteins[1] (Figure 1-1). Among the nanostructures which are increasingly being used as biological sensors and delivery vehicles for therapeutic agents, nanoparticles are of particular interest. While the material makeup of nanoparticles used in biomedical applications are diverse, this project will focus on the semiconductor and noble metal materials. Beneficial characteristics of such nanoparticles include tunable size, shape, and functionality attributes; relative ease of fabrication
via ‘wet chemistry’ techniques[2]; large surface area-to-volume ratio; simple surface functionalization (via sulfur-metal linkages on noble metal nanoparticles) [3-4], and enhanced stability of attached macromolecules such as nucleic acids[4-5].

Semiconductor and metal nanoparticles, typically in the range of 1-100nm in diameter, are attractive due to their unique electronic and photonic properties [2, 6-7]. Small semiconductor (commonly ZnS, CdS, CdSe) nanoparticles, known as quantum dots, have met with tremendous success as biological labels, due to their tunable fluorescent properties[8]. Noble metal (Au, Ag, Pt, and Cu) nanoparticles have accrued interest owing to a phenomenon unique to these nanomaterials, known as localized surface plasmon resonance (LSPR). LSPR occurs when a small spherical metal particle is subjected to an oscillating electric field, which causes the conduction electrons at the metal surface to oscillate collectively in response[7]. The local field enhancements attributed to surface plasmons have paved the way for many surface-enhanced spectroscopy techniques, including surface-enhanced absorption, fluorescence, photochemistry, and Raman scattering (SERS)[9]. These techniques have applications in chemistry, biology, and medicine.

Gold (Au) and silver (Ag) nanoparticles have been used extensively as biological sensors which take advantage of plasmon resonance to enhance detection of specific targets. Noble metal nanoparticle-based sensors benefit from the extreme sensitivity of the localized surface plasmon resonance (LSPR) spectra to environmental changes[10]. Application of metal nanoparticles is not limited to molecular detection: recently, gold nanoparticles (GNPs), and to a lesser extent silver nanoparticles (SNPs), have been harnessed as delivery vehicles for therapeutic agents, including antisense oligonucleotides[11-13] and other small molecules[4, 14-15]. Small metal nanoparticles offer many advantages as drug carriers, including high-density surface ligand
attachment, transmembrane delivery without harsh transfection agents[11], protection of the attached therapeutic from degradation[5, 12, 16], and potential for improved timed/controlled intracellular release[16]. The photophysical properties of noble metal nanoparticles[17] may potentially bring these materials to the forefront of drug delivery, enabling targeted delivery, spatiotemporally controlled (photo-)release, and delivery confirmation via imaging.

**Figure 1-1 - Nanoparticle-Biomolecule Hybrids**
(based on figure from Katz, Angew. Chem., Int. Ed. 2004, 43, p.6042)

### 1.2.1 Localized Surface Plasmon Resonance (LSPR)

Localized surface plasmons (LSPs) are excitations of the conduction-band electrons of sub-wavelength conductive nanoparticles coupled to an incident electromagnetic field (i.e. light)[18]. Whereas noble metals in bulk form have overlapping conduction and valence electron bands, metal nanoparticles, consisting of a number of electrons large enough to be characterized by properties differing from those of single atoms, yet too few electrons to reflect the properties
of the bulk metal, have close lying (not overlapping) conduction and valence bands between which electrons to move relatively freely based on their energy state[17] (Figure 1-2). Surface plasmons are based mostly upon the oscillations of free electrons within the conduction band that occupy energy states immediately above the Fermi level[17, 19], where the Fermi level is the outer boundary of electron energy states at absolute zero temperature. Electrons which exist in energy states above the Fermi level are available for conduction. The optical properties of noble metal nanoparticles are mostly influenced by the said electron oscillations, however, the movement of bound electrons can also contribute to the plasmon spectra of these resonant particles[7]. Localized surface plasmon resonance (LSPR) occurs as the charge-density oscillations of the conduction electrons on the surface of metal nanoparticles[6] attain resonance conditions. Resonance modes arise due to the fact that the curved surface of the nanoparticle exerts an effective restoring force on the oscillating electrons[18]. Under resonance conditions, the nanoparticle acts as an electric dipole, resonantly absorbing and scattering electromagnetic fields[18]. In 1908 Mie applied Maxwell’s equations to sub-wavelength conductive spheres (Mie Theory[19]) in order to describe the optical properties of metallic nanoparticles. Mie theory describes and quantifies the extinction spectra (the combination of absorption and scattering) of spherical particles of arbitrary size in various dielectric media[7]. Since the advent of Mie theory, other models have been developed to further and more precisely describe the electronic properties of metal nanoparticles, especially non-spherical particles[20]. However, Mie theory remains an important model for describing the size-dependent optical properties of spherical particles in solution.

Localized surface plasmons (LSPs) effect strong absorption and scattering of light. This phenomenon is the underlying cause of the bright colors observed in solutions of colloidal noble-
metal nanoparticles. Gold (Au) and silver (Ag) nanoparticles have properties such that their plasmon resonant absorption spectra lie within the visible region[17]. Colloidal Au nanoparticles are characterized by a wine-red color, with a plasmon band centered around 520nm[21]. Colloidal Ag nanoparticles are characterized by a yellow/green color, with a plasmon band around 400–420nm dependent on particle size[22]. The plasmon frequency of these particles depend on particle size, shape, surface state, interparticle distance, and the surrounding dielectric environment[6], as well as adsorbed species on the particle surface. The plasmon frequency dependence on interparticle distance has been utilized in colorimetric assays to determine hybridization-induced coupling of DNA-functionalized gold and silver particles[10, 23-24]. The plasmon frequency dependence on particle size and shape has lead to tailoring of nanoparticle photophysical properties for specific applications via various synthesis methods and conditions[25].

According to Mie Theory, the extinction cross-section ($C_{ext}$) of metal nanoparticles is comprised of both absorption ($C_A$) and scattering ($C_S$) components. For small metal particles ($\lambda >> 2R$ where $R$ is the particle radius), the extinction cross-section is dominated by dipole oscillations at the particle surface[26-27], leading to a dipole approximation of extinction based on Mie Theory[6, 19]:

$$C_{ext} = \frac{9V\varepsilon_m^{3/2}}{c} \cdot \frac{\omega \varepsilon_2(\omega)}{[\varepsilon_1(\omega) + 2\varepsilon_m]^2 + \varepsilon_2(\omega)^2}$$  

where $V$ is the spherical particle volume, $c$ is the speed of light, $\omega$ is the angular frequency of the incident light, $\varepsilon_m$ is the dielectric constant of the surrounding medium, $\varepsilon_1$ and $\varepsilon_2$ are the real and imaginary parts of the dielectric function of the particle metal, respectively, where $\varepsilon_{particle} = \varepsilon_1(\omega) + i\varepsilon_2(\omega)$. The optical extinction reaches a maximum amplitude at the plasmon resonance
condition, or when $\varepsilon_1(\omega_{\text{res}}) \approx -2\varepsilon_m$. The plasmon resonance frequency describes the location at which absorption and scattering (both components of extinction) of light by metal nanoparticles are at a peak value based upon maximum amplitude of electron oscillations and interactions. The extinction cross section of a 60nm silver colloid has been reported as $1.4 \times 10^{-10}$ cm$^2$, which may be compared to the cross-section of fluorescein, a standard strongly absorbing and bright fluorophore, which has a value of $\sim 2.0 \times 10^{-15}$ cm$^2$[28]. The strong optical properties of metal nanoparticles compared to other well-known chromophores increases interest in their use in light scattering and surface-enhanced spectroscopy applications.

For larger nanoparticles ($2R$ comparable to incident wavelength $\lambda$) the dipole oscillation mode is joined by higher multipole modes. The sum of these plasmon modes are described by the more robust complete Mie theory. For small nanoparticles, the absorption component of extinction predominates, based on nonradiative processes of plasmon decay[29]. As metal nanoparticles increase in size, the scattering component begins to contribute significantly to the total extinction, based on radiative emission of electromagnetic energy by the surface plasmon[29]. Thus, for applications where strong scattering of incident light (radiative coupling to outgoing electromagnetic field[29]) is desired, such as for surface-enhanced fluorescence or photochemistry, the use of larger metal nanoparticles ($2R > 30$nm)[6-7] may be beneficial. As previously stated, surface plasmon resonance frequency is largely dependent on particle size, and larger particles, dominated by multipole absorption and scattering, typically exhibit red-shifted (longer wavelength) extinction spectra[26]. As a result of these phenomena, the extinction spectra of metal nanoparticles, their absorption and scattering properties, may be tailored via control over particle size and shape.
1.2.2 Surface-Enhanced Spectroscopy and Photochemistry

Localized surface plasmons effectively confine incident light into sub-wavelength (nanometer-scale) dimensions[30]. This condensing of incident light produces large local enhancements of electromagnetic fields. Along with E-field enhancement, enhanced absorption and scattering of light by nanoparticles at the surface plasmon resonance (SPR) frequency are observed [27, 29]. The special electronic properties of metal nanoparticles and the influence of these electronic properties on molecular species on or near the particle surface constitute the basis of surface-enhanced spectroscopy techniques. These include surface-enhanced absorption, surface-enhanced Raman spectroscopy (SERS), surface-enhanced fluorescence (SEF), and surface-enhanced photochemistry[9]. The amplification or quenching of signals near a metal
nanoparticle surface arises due to a concentration and enhancement of electromagnetic energy in the region of space on and just outside the metal sphere surface[9, 31]. Enhancements in absorption, fluorescence, and Raman scattering can be based upon enhancement of absorption processes of compounds near the metal surface, provided that the molecular absorption bands for the compounds in question are close in frequency to the SPR frequency of the metal particles[9, 32]. Alternatively, enhancement of emission processes from excited chromophores near the surface of metal nanoparticles may be responsible for overall enhancement of fluorescence[33] and luminescence signals. The surface-enhanced resonance Raman scattering (SERRS) technique, often used for sensitive single-molecule detection, is based upon the fact that Raman-scattered light, thus detection signals, can be significantly magnified by adsorbing target molecules onto roughened metal surfaces or metal nanoparticles[34]. Surface-enhanced fluorescence (SEF) on silver (Ag) particles and films has been observed and extensively studied. SEF, as well as most other surface-enhanced spectroscopy processes, is dependent on particle size[31] and on distance between target molecules and the metal particle surface, where emissions from molecules directly on the metal surface are often quenched vs. enhanced.

In addition to the enhancements of Raman scattering, light absorption, and fluorescence observed as result of interaction of molecular targets with metal nanoparticles, enhanced photochemistry has been predicted[35-36] due to observed increases in molecular absorption cross-sections of photo-responsive molecules near metal surfaces (thin films[37], rough surfaces[36, 38], and nanoparticles[39]). Surface-enhanced photochemistry[32] has potentially significant applications in chemistry and biology[9], of note for the studies proposed herein, whereby photo-catalyzed reactions and photo-induced release of therapeutics from metal nanoparticles will be characterized. Surface-enhanced photochemistry may yield unique
pathways for photoexcitation and photoreaction[32], with a wide range of applications. Reports have been made of enhancements of photo-induced reactions of aromatics adsorbed on silver surfaces (rough metal and nano-scale surfaces), as well as enhancement of 2-photon decomposition[36]. Although surface-enhancement of photochemistry has been predicted and observed in various cases, proximity to a metal nanoparticle surface may in some cases serve to quench photochemical reactions, as predicted by Nitzan et al[35]. However, many reports of generation of excited states in photoreactive moieties on metal nanoparticles sufficient to effect photo-induced chemical reactions[17, 40] provide promising evidence for the potential of surface-enhanced photochemistry.

The enhancement factor of photochemistry at or near a metal surface, related to enhanced absorption and excitation, will depend on particle shape and size, as well as the distance of the photoactive molecule from the particle surface[36]. The size factor, as depicted by results produced by Zhang et al in studies on field-enhancement by silver nanoparticles[31], will be considered in proposed studies for the choosing of appropriate SNP sizes for maximum enhancement of photochemical processes. Moderately sized particles (around 50nm in diameter) have been found previously to display the most enhanced intensity of local fields and SEF[31]. The distance factor is of particular importance in relation to photochemical processes of molecules directly adsorbed or tethered a short distance from the metal surface (<1nm), where the energy-transfer rate to the metal surface surpass the photochemical reaction rate. In such cases, the effective transfer of energy from the excited molecule to the metal surface competes with the enhanced accumulation of energy by the molecule due to interaction with surface[41], thus quenching the molecule’s excited states. Thus an important consideration in surface-enhanced photochemistry will be tailoring the distance of a photoactive molecule/functional
group from the metal nanoparticle surface, in order to strike a compromise between field enhancement (i.e. enhanced photochemistry) and nonradiative damping[9] or quenching.

1.2.3 Optical Properties of Silver Nanoparticles

Gold (Au) and silver (Ag) nanoparticles in the diameter range of ~2-100nm exhibit SPR spectra in the visible region, which are tunable and dependent on particle shape, size, environment, and interparticle distance. While gold nanoparticles have been used extensively in many of the applications previously discussed, silver nanoparticles have unique properties which make them a desirable alternative particle type in many cases. Silver nanoparticles (SNPs) are the strongest light scatterers of the noble metal particles[22, 42]. The intense light absorption and scattering (extinction) band of silver nanoparticles (~380-420nm dependent on particle size) is due primarily to free conduction electron oscillations, although bound electron movements also contribute to the optical spectra[22]. The light scattered by an Ag colloid has the same wavelength of the incident light, as electrons emit only by transition to their original ground state[22], vs. relaxing into other vibrational states before returning to their ground state (process of fluorescence). Thus enhancement of absorption/emission of light by molecules near the silver particle surface is dependent on particle size and proximity or overlap of the resonance (SPR) spectra with the absorption/emission bands of the molecular species.

The light absorption and scattering properties for silver nanoparticles of different sizes have been calculated in accordance with Mie Theory[19, 22] (Table 1-1). For larger particle sizes (~50-60nm) as will be seen in the current studies on SNPs for nucleic acid delivery and photo-controlled release, scattering efficiency ($Q_{sca}$) is high ($\approx$5), where

$$Q_{sca} = \frac{C_{sca}}{\pi r^2}$$

2
Where values >1 indicate the ability of a particle to scatter photons outside its physical cross-sectional area[22]. Thus silver particles in this size range may scatter light (as well as enhance electromagnetic fields) at or above the physical metal surface. The scattering cross section of a silver particle is about 10 times greater than that of a gold particle of the same size[22]. Silver nanoparticles in the diameter range of 40-70nm (Table 1-1) achieve high scattering efficiencies while maintaining surface plasmon resonance in the UV to visible range appropriate for traditional and red-shifted photocleavable compounds typically used as photo-caging compounds.

The light-scattering and field-enhancement properties of silver nanoparticles have led to an increased interest in their use as sensors, biological labels, and substrates for surface-enhanced absorption, fluorescence, and photochemistry. Enhanced photochemistry has been predicted on silver surfaces, and silver nano-materials comprise the best enhancing substrate found to date[35]. Silver nanoparticles have high extinction coefficients[22, 36] along with low SPR frequencies (near-UV range) which make these particles particularly appropriate for studies of enhanced photochemistry of photoreactive moieties sensitive to UV irradiation.

Table 1-1 Calculated light absorption and scattering properties for SNPs of different sizes[22].

<table>
<thead>
<tr>
<th>Dia (nm)</th>
<th>( \lambda_{\text{max}} ) (nm)*</th>
<th>( C_{\text{sca}} ) (cm²)</th>
<th>( I_{\gamma}(0) )</th>
<th>( I_{\gamma}(90) )</th>
<th>( \epsilon ) (M⁻¹ cm⁻¹)</th>
<th>( \eta )</th>
<th>( Q_{\text{sca}} )</th>
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<td>3.03 × 10⁹</td>
<td>1.05 × 10⁹</td>
<td>8 × 10¹¹</td>
<td>0.877</td>
<td>3.82</td>
</tr>
</tbody>
</table>

* Diameter range of 40-70nm.
1.2.4 Nanoparticle Synthesis

A variety of methods are available for the production of nanoparticles within a specified size distribution[2]. These include reduction of metal ions via chemical capping agents to form metal nanoparticles in solution, vacuum deposition, electron-beam (EB) lithography, laser-ablation (photo- and radio-lytic reduction of metal ions), and electrodeposition (electrochemical deposition of nanoparticles)[6]. Spherical noble-metal nanoparticles for applications such as delineated herein are commonly prepared via ‘wet chemistry’ procedures, where ‘clusters of metal atoms… are formed in the presence of a surface-capping ligand’[2]. The capping ligand[2], whether a hydrophobic ligand, a charged ligand, or a polymer stabilizing agent, serves to stabilize individual nanoparticles and prevent aggregation via particle-particle repulsion. The wet chemistry synthesis procedures are often simple and practical in the tunable synthesis of spherical particles of sufficiently narrow size distribution.

Au and Ag nanoparticle colloids are often prepared via reduction of metal ions (reaction of HAuCl₄ or AgNO₃ in aqueous solution) via sodium borohydride, (NaBH₄) or trisodium citrate[25, 43]. The ratio of capping agent to metal in solution during reduction may be varied to achieve a desired size distribution (where higher a high ratio of capping agent to metal will produce smaller particles). Reduction with citrate has been reported to produce colloids with more uniform size distributions, greater stability in solution over time, and more pronounced surface-enhanced optical properties than colloids synthesized via borohydride-reduction[44]. Nanoparticles formed via citrate-reduction are coated by negatively charged electrical double layers which provide stabilization of individual Au or Ag nanoparticles[6]. Wet chemistry synthesis of silver and gold nanoparticles via citrate-reduction has been adopted herein due to
ease of synthesis and simple replacement of capping ligand (citrate) with thiol-modified biomolecules at the particle surface (Figure 1-3).

**Figure 1-3** - Citrate-stabilized SNP functionalization with thiol-modified biomolecule (DNA)

### 1.2.5 Nanoparticle Characterization

A myriad of techniques exist for the characterization of metal nanoparticles. The optical properties of noble metal nanoparticles may be determined theoretically[20, 22] and experimentally via UV-Visible spectroscopy and microscopy techniques[42]. Particle morphology, including shape and size, may be determined to some extent via spectroscopy techniques using the fact that shape and size affect SPR spectra, but are more often determined via electron microscopy techniques[42].

#### 1.2.5.1 Characterization Technique: TEM

TEM (Transmission electron microscopy) is an analytical tool for determining the shape, size, and arrangement of nanomaterials. TEM produces images based upon electron interactions with a thin sample specimen, where more or less dense regions of the specimen produce the imaging contrast portrayed by the final photographic films. Several studies have utilized TEM as
a characterization method for different nanoparticles, including SNPs[45]. TEM will serve as a characterization tool herein, in order to determine morphology and size distribution of citrate-reduced silver colloids.

1.2.5.2 Characterization Technique: UV-Visible Spectroscopy

UV-visible spectroscopy is a very simple and quick method for characterizing colloidal metal nanoparticles based upon their optical (light-absorption) properties. It is not an ideal technique for providing detailed information regarding NP shape and size distribution when compared to microscopy-based analysis techniques, but is a semi-quantitative method for determining the presence, approximate size, and extent of aggregation of nanoparticles in solution. The determination of such is based upon the plasmon resonant properties of the metal nanoparticles. The bandwidth, peak height, and maximum absorption of a UV-Vis spectra for a particle colloid are dependent on individual particle size, size distribution (bandwidth), surface state, surface coverage (can induce blue- or red- shifting of spectra), interparticle distance, and surrounding dielectric environment[6]. Absorbance spectroscopy can also serve as an indicator of material deterioration and is a qualitative method for indicating whether a given colloid is still within its usable lifetime for biomolecule functionalization. Absorbance spectroscopy has been carried out herein using an LS55B Luminescence Spectrophotometer (Perkin Elmer, Boston, MA).

1.2.5.3 Characterization Technique: Dynamic Light Scattering

Dynamic light scattering (DLS) is a technique by which the size distribution of small particles in solution (a colloid) may be quantified from changes in a light field (frequency shifts, polarization changes) due to its interaction with the colloidal system[46]. The information gathered by DLS is related to the light scattering properties of suspended solutes. Dynamic light
scattering may be used to measure the diffusion coefficient for solute molecules/particles, which is related to the radius of a spherical molecule/particle (Stokes Einstein equation):

\[ D = \frac{k_b T}{6\pi \eta r} \]

Where \( k_b \) is the Boltzmann constant (in J K\(^{-1}\)), \( D \) is the diffusion coefficient (in m\(^2\)s\(^{-1}\)) and \( T \) is the temperature in Kelvin, and \( r \) is the hydrodynamic (or Stokes) radius. The hydrodynamic radius is the apparent radius of a molecule/particle in solution, based on their measured rates of diffusion. Dynamic light scattering analysis of a colloid also provides a measure of the polydispersity (size distribution) of the particle solution.

Dynamic light scattering instruments may also be used to measure zeta potential (electrokinetic potential at the effective shear plane between the moveable and non-moveable part of the double layer[47]) of colloids. Zeta potential of solutes may be determined from the light scattering properties of molecules/particles moving within an applied electric field (electrophoretic light scattering)[47]. Zeta potential is an important indication of colloid stability and biomolecule adhesion onto the particle surfaces. DLS will been performed herein using a Malvern ZetaSizer Nano ZS (Malvern, Worcestershire, UK).

1.3 Metal Nanoparticle Functionalization with Biomolecules: Nanohybrids

Recent advances in metal nanoparticle synthesis and functionalization with various organic compounds, including proteins, nucleic acids, long-chain hydrocarbons, have led to increasing applications of noble metal nano-hybrids in biology and medicine. These nano-hybrids incorporate the bioactive properties (catalytic functions, recognition and binding properties) of macromolecules with the unique optical and photochemical properties of noble metal nanoparticles[2]. The relative ease of noble metal nanoparticle functionalization stems
from a variety of means for immobilizing biomolecules on the metal surface. These include electrostatic interactions as well as covalent binding\[2\]. Metal nanoparticles that are synthesized by salt reduction are in most cases stabilized by anionic ligands such as carboxylic acid derivatives\[2\] (notably citrate), which give the metal particles a net negative surface to stabilize the particles and prevent particle-particle interactions (aggregation). The negative surface allows electrostatic interactions of cationic ligands/functional groups with the metal particle surface, for example the positively charged amino acid side chains of proteins\[2\]. Predominantly, functionalization of metal nanoparticles is achieved via covalent coupling of biomolecules to the metal surface. The binding of thiols to the metal surface (via covalent sulfur-metal bond), is a predominant method of nanoparticle functionalization\[2\]. Amine and carbonyl functional groups may also interact with the metal surface\[48\] and serve to tether biomolecules to the nanoparticle surface using standard conjugation chemistries.

The functionalization of metal nanoparticles with nucleic acids is of particular interest in the current studies. Nucleic acid-nanoparticle hybrids have been used for a variety of applications, including DNA detection based on LSPR absorbance changes\[6, 49\], particle assembly based on hybridization\[10, 21, 23-24\], DNA intracellular delivery\[3-4, 11, 15, 50\], and gene therapy\[12-13\]. Nucleic acids are typically immobilized on metal nanoparticles via covalent coupling (thiol end-modified nucleic acids). The nucleotide bases of single-stranded DNA (ssDNA) are also available for binding to the metal surface via amine and carbonyl functional groups\[48, 51\]. Double-stranded DNA (dsDNA), however, generally does not interact with anionic ligand stabilized nanoparticles due to repulsive forces between the negatively charged particles and the phosphate backbone of the DNA (Figure 1-4). In cases where thiol linkage of nucleic acids is desired over ‘nonspecific’ base interactions with the metal surface\[49, 52\],
techniques must be employed to create an environment where sulfur-metal bonds are favored over other interactions. Thiol linkage predominance serves to increase surface coverage due to tight packing of ligands on the particle surface. Such techniques will be investigated in the proposed studies on antisense oligonucleotide functionalized silver nanoparticles.

![Figure 1-4 - Nucleic acid interactions with metal nanoparticles](image)

1.3.1 SNP Functionalization with Nucleic Acids

Metal-biomolecule nano-hybrids are often developed by assembling single molecule monolayers, or mixed monolayers (assembling a monolayer of one type of biomolecule and backfilling with another), on the nanoparticle surface via functional groups which interact with metal, such as thiols, amines, carbonyls, etc[53]. Oligonucleotides may be end modified with thiol functional groups in order to assemble a tightly-packed monolayer of nucleic acid on a nanoparticle surface. Following thiol adsorption to the metal surface, the nucleic acids will ideally be oriented perpendicularly to the surface. Oligonucleotide bases also have intrinsic reactive functional groups (significantly amines) which have the tendency to associate with metal surfaces. While these ‘non-specific’ interactions are not desired in the final nanohybrids, they
may serve to stabilize SNP-oligo conjugates in the early stages of functionalization[52]. Typically nanoparticle-nucleic acid conjugates must be slowly adjusted to physiological salt concentrations ($\approx 150$ mM), conditions which serve to minimize the electrostatic repulsion between individual oligonucleotides (‘screening effect’) [54] that arises due to the negatively charged state of the phosphate backbone. This screening will enhance surface coverage [10, 52, 55-56], as well as create an environment at the particle surface most favorable to ligand attachment via thiol bonding vs. amine, carbonyl, or other functional group interactions. The screening effect will also apply to the negatively charged (citrate-stabilized) nanoparticle surface, and without the presence of a stabilizing layer of biomolecules, will effect irreversible aggregation of the colloid [10, 51]. Thus the required level of salt (typically NaCl) must be added gradually, and a fine balance must be struck in order to maximize surface coverage as well as colloid stability. Nanoparticle size (curvature) [57], oligonucleotide length and sequence [48], presence of ‘linkers’ or spacers molecules, presence of low levels of surfactant [56], and final salt concentration have all been found to affect surface coverage and stability of SNP-oligo conjugates.

**1.3.2 SNP Functionalization: Solutions for a Difficult Process**

The functionalization of silver nanoparticles has been found to be exceptionally difficult, and requires delicate equilibration to final salt concentration, or ‘salt-aging’ [10], and often special design of attachment moieties [58]. Functionalized SNPs are typically unstable and prone to chemical degradation [59]. Gold nanoparticles are not as sensitive, which has led to the more widespread use of these particles in sensing and medical applications. However, as previously described, silver nanoparticles have special properties which make them highly desirable as an alternative to gold nanoparticles in many applications. Thus the optimization of silver
nanoparticle functionalization, and the development of novel methods for increasing colloid stability during functionalization and salt addition are desired. Such methods include the use of mild surfactants such as sodium dodecyl sulfate (SDS) or Tween 20 during the ‘salt-aging’ process[48], and according to a method developed herein the use of a small amine-containing molecule, Tris, which interacts with metal surface. Tris(hydroxymethyl)aminomethane (Tris) is a compound often found in buffer solutions for nucleic acids. Although the presence of Tris during nucleic-acid attachment to metal surfaces has been shown previously to not significantly affect ligand coverage on gold nanoparticles[56], a custom Tris buffer-based functionalization method has been shown to dramatically enhance silver nanoparticle-conjugate stability during functionalization, as well as produce high oligonucleotide surface coverage. This has far-reaching implications in silver nanoparticle functionalization, and may serve to broaden SNP-biomolecule conjugates applications in biotechnology and medicine.

1.4 Nanoparticles as Drug Delivery Vehicles

Functionalized metal nanoparticles have accrued significant interest as drug delivery vehicles and potential substrates for targeted and controlled release of therapeutic agents in vivo. Protein-based and, of interest herein, nucleic acid-based drugs are in need of novel delivery technologies which can enhance delivery, prolong lifetime, enable precise targeting, improve efficacy, and minimize side effects[16]. Nanoparticles provide a promising and exciting solution, bringing to the table improved delivery and stability characteristics, as well as the potential for external control (thermal, photo-thermal[4], light-activated[60]) over drug release and activity in vivo[16]. Various nanoparticle formulations have been used for delivery of biomolecules such as small molecule therapeutics[14, 16, 61-62], proteins[16, 62], and nucleic acids[3, 11-13, 15-16, 50, 63]. Nanoparticles have been shown to protect immobilized biomolecules from degradation
(notably nucleic acids[5]), thus prolonging their active lifetime[16]. Protection of nanoparticle-immobilized nucleic acids from degradation by nucleases has been partially attributed to the high local salt concentration which accompanies dense nucleic acid packing on the particle surface. High salt concentration is known to deactivate the enzymatic activity of DNase II[64], a principal agent of nucleic acid degradation in vitro. Other explanations for the stability of nucleic acids when attached to nanoparticles include steric hindrance of nucleases, protection of a surface-attached 3’ oligonucleotide end from 3’ exonucleases[65], and possible protective layers of positively-charged serum proteins which absorb to the highly-charged nucleic acid-nanoparticle surface[11].

Biomolecule surface-functionalized nanoparticles not only possess characteristics of enhanced stability, but provide a means to deliver greater amounts of target therapeutic via high density surface coverage, often without requiring additional transfection agents for passage through the cell membrane[11]. Backfilling of a nucleic-acid functionalized nanoparticle with lipid or polymer species (for example polyethylenimine or PEI) often used for gene delivery can further enhance transfection efficiency beyond that of lipid or polymer transfection agents unattached to nanoscale carriers[4, 66]. Nanoscale delivery vehicles have been shown to enhance cellular uptake via facile endocytosis of small functionalized nanoparticles[62], intracellular trafficking of delivered therapeutics, and nuclear access[67], the later being an important process in gene expression based therapies. Beyond the their enhanced cellular uptake and their delivery of high drug concentrations, metallic nanoparticles are also attractive due to the possibility of controlling the release of the bound therapeutic molecules from the particle surface. The ability to attach ligands via specific interactions to the metal nanoparticle surface offers a high degree of engineering precision[16] with the potential for ligand/receptor targeted delivery[15] and
controlled release of the particle payload. Controlled release mechanisms of drugs from nanomaterial surfaces have included thermal, pH, chemical, and ionic strength stimuli based processes. The optical and photophysical properties of metal nanoparticles further provide unique prospects for photo-controlled release and characterization of delivery and release based on SPR-enhanced imaging signals.

1.5 Photo-controlled Release

Light-responsive systems are of great interest in the field of drug delivery and gene therapy, owing to the capability of external, spatiotemporal control (in time and location) over the delivery and activation of therapeutics coupled with such systems. Light-responsive drug delivery systems are triggered by electromagnetic radiation, typically in the UV, visible, and near infrared (NIR) range[68]. These systems are based upon photosensitive compounds which can be incorporated into a drug delivery vehicle, or coupled to the drug itself (‘caging’ compounds), and may switch to an active or inactive state upon electromagnetic irradiation within a specific frequency range. Caged compounds, ‘whose activities are suppressed by the covalent linkage of photocleavable protecting groups but are restored upon photo-irradiation’ are powerful tools for spatiotemporal control over drug activity in living systems[69]. Photocleavable groups have been used to cage, or inactivate, various biomolecules, including nucleotides, proteins, and nucleic acids[69-71], for the purpose of controlled, on-site photo-activation. Uncaging via light irradiation allows rapid, spatially and temporally defined release of a biomolecule at intended tissues or even within a specific intracellular compartment[69].

1.5.1 Potential of Nanoparticle-Based Caged Compounds

Caging of nucleic acids has been employed for many applications in biology and medicine, including controlled delivery, gene therapy, and analysis of gene expression[72].
Nucleic acid drugs depend on hybridization-based function, thus caging of sites along an oligonucleotide (backbone phosphates, base functional groups) which prevent hybridization to a complementary oligonucleotide effectively inactivate these therapeutics. While the caging of nucleic acids for light-activated release and gene therapy has been achieved by a variety of methods, mostly via direct modification of nucleic acids with photo-labile protecting groups which prevent base-pairing with a complementary sequence[69-71, 73], recent interest lies in the use of nanoparticles as ‘bulky’ caging groups, which can suppress the activity of ‘cargo’ biomolecules until release of the later from the nanoparticle surface, as demonstrated by Nakanishi et al in the release (uncaging) of histamine from gold nanoparticles[74]. The dense packing of nucleic acids on metal surfaces has been shown to not only enhance stability of nucleic acid therapeutics by preventing nuclease degradation[5], but also reduce hybridization via steric and electrostatic hindrances[52]. Nanoparticles with photo-responsive moieties may provide enhanced external control over intracellular release and activation of cargo therapeutics[68], while also providing a solid support for enhanced stability and delivery of these therapeutics[74] prior to on-site release. Photo-responsive nanoparticles, consisting mostly of gold and other metal or semiconductor materials, have been used in the delivery and spatiotemporal activation of anticancer drugs[14, 61], cell signaling molecules[74], and nucleic acids[60].

While increased interest has arisen in the use of nanoparticles as delivery vehicles, controlled release of DNA from surfaces is a relatively unexplored area[75], albeit a promising one. Controlled release of nucleic acid therapeutics in functional form from solid-surface carriers, which may enhance stability, delivery, imaging, and provide sensitive external control, is of great interest in gene therapy[75]. Release of nucleic acids from surfaces (including
nanoparticle surfaces) via thermal, pH[76], ionic strength, and chemical stimuli[77] have been reported, but the integration of photoresponsive units into such systems offers the capability for regulated release with higher spatiotemporal specificity. Properties which define good caging systems include high rate of release following photo-exposure, precise focusing of release location based upon ability to focus incident light (focusing at organ, tissue, cellular, or sub-cellular compartment levels[69]), external control over time of release and activation of delivered therapeutics, and tunable amount of release based upon degree of light exposure[69]. The design of a good caged system must take into account the following ideals: high absorption and quantum yield properties of the activating photochemical reaction, harmless byproducts, a water soluble form capable of being easily delivered across the cell membrane, a low level of activity (‘leaking’) prior to photoactivation, and photoreactivity at wavelengths above 300nm, considering the damaging effects of lower wavelength light on biological tissues and DNA itself. Noble metal nanoparticles are presented as a potentially superior alternative to traditional carrier vehicles and caging systems, providing ameliorated delivery properties as compared to naked nucleic acids or other transfection agents alone[16, 66] and the potential of surface-enhanced photochemistry, including enhanced absorption and potentially enhanced photocleavage at SPR peak wavelengths (~400-420nm for silver nanoparticles). Nanoparticle-assisted photocleavage of traditional caging compounds at higher wavelengths than the traditional UV-range (300-350nm) would be of enormous interest in drug delivery and gene therapy. In recent years efforts have been made to create photolabile protecting groups with enhanced photoreactivity at red-shifted wavelengths, for example modified 2-nitrobenzyl derivatives with enhanced one-photon and two-photon photoreactive properties[78]. Achieving efficient reaction at longer wavelengths is difficult due to decreased energy of individual photons at longer wavelengths, and traditionally
low extinction coefficients of photolabile compounds at red-shifted wavelengths. Surface plasmon resonant nanoparticles with exceptional optical and electronic properties may allow more efficient photoreactivity of traditional photolabile compounds due to the concentration of the electromagnetic field (i.e. light) in the vicinity of the particle surface (Figure 1-5) [79]. Figure 1-5 shows the field lines, excluding those scattered, surrounding a small \((x > 1)\) metallic sphere with a size parameter \((x)\) of 0.3, where the particle circumference divided by the wavelength of incident light \((2\pi a/\lambda)\). The corresponding size parameter of a 50nm diameter silver particle irradiated with light at 400nm would be approximately 0.4.

![Figure 1-5](image)

**Figure 1-5** – Electromagnetic field lines around a small metallic sphere illuminated by light in the UV/Visible range. Dashed vertical line indicates the effective size of the sphere for absorption of incident light[79].

A limited number of reports have been published of photo-responsive metal nanoparticles for drug delivery, attributable to the belief that a small metal core may strongly deactivate excited states of photoactive units close to the plasmon resonant surface[75]. However, preservation or metal-enhancement of photochemistry at the nanoscale, for properly designed
metal nanoparticle systems, has been both predicted\cite{36} and reported\cite{40, 60, 80}. Conditions including particle composition, particle size, distance between plasmon resonant surface and photo-responsive group, and other properties of photoactive unit itself, need to be precisely designed and controlled to allow surface-enhanced photochemistry vs. competitive quenching\cite{41}. Hu et al\cite{40}, with their work on gold clusters, reported that ‘it is possible to generate sufficiently long-lived excited states in SAMs (self-assembled monolayers on colloidal metal) functionalized with photoreactive moieties that effective photochemical modification of the metal-dielectric interface can be observed.’ Nitrobenzyl derivatives on nano-gold surfaces have been shown to exhibit similar photoreactivity to that observed in solution phase\cite{17}, and o-nitrobenzyl groups on gold have been used as a means to effect photo-release of electrostatically complexed nucleic acids\cite{60}. While most studies of photo-responsive nanoparticles have centered around gold-based materials, silver nanoparticles offer higher extinction coefficients and blue-shifted plasmon resonant peaks which make them a most appropriate alternative to gold nanomaterials for surface-enhanced photochemistry and photo-controlled drug delivery. The studies undertaken herein will seek to explore the photochemical properties of oligonucleotide-loaded photo-responsive silver nanoparticles.

1.5.2 Phocleavage Reaction Scheme

Reviews of currently available photolabile ligands have been published\cite{81}, which may allow selection of a compound most appropriate for a specific caging application. A caging compound will ideally possess high extinction coefficient and quantum yield properties at wavelengths which are not detrimental to biological systems. The quantum yield ($\phi$) of a photochemical reaction is a measure of the efficiency with which absorbed light (incident photons) produces photolysis, where unity indicates 100% efficiency. The absorptivity of a
compound at a particular wavelength of incident light, in other words the likelihood that a photon will be absorbed[69], is quantified in the compound’s extinction coefficient ($\varepsilon$, units: M$^{-1}$ cm$^{-1}$). Beer’s Law may be used to find the extinction coefficient of a photochemical compound based on absorbance measurements:

$$A_\lambda = \varepsilon C l$$  \hspace{1cm} (4)

Where $\varepsilon$ is the extinction coefficient, $C$ is the concentration of the absorbing molecule, and $l$ is the optical pathlength. Extinction coefficient known, a functional quantum yield may be calculated experimentally for a photochemical reaction:

$$\phi = \frac{1}{I \varepsilon t_{90\%}}$$  \hspace{1cm} (5)

Where $\varepsilon$ is the decadic extinction coefficient in cm$^2$/mol (or $10^3$ x M$^{-1}$ cm$^{-1}$), $I$ is the irradiation intensity (moles of photons cm$^2$ s$^{-1}$), and $t_{90\%}$ is the irradiation time (in seconds) for 90% conversion of the photocleavable compound.

While many photocleavable compounds exist for caging of biomolecules, 2-nitrobenzyl derivatives are some of the most widely used[69]. A 2-nitrobenzyl protecting group was used in the first caging of adenosine 5'-triphosphate (ATP) by Kaplan et al[84], with a reported maximum quantum yield of 0.63. 1-(2-nitrophenyl)ethyl phosphate esters have been reported to demonstrate high quantum yields (0.49-0.63) in applications of caged nucleotides[85-86]. A 1-(2-nitrophenyl)ethyl (NPE)-based photocleavable (PC) linker available commercially through Glen Research (Sterling, VA) and IDT (Integrated DNA Technologies, Coralville, IA) has been used for immobilization onto solid surfaces, purification, and characterization of nucleic acids[87]. This PC linker demonstrates high photocleavage efficiency under UV-irradiation (300-350nm)[88], and has been chosen as the photo-labile group herein for immobilization and
release of oligonucleotides from a metal nanoparticle surface. Upon photo-irradiation, the phosphodiester bond between the photocleavable group and the phosphate is cleaved, resulting in the formation of a 5’-monophosphate on the released moiety (oligonucleotide)[87]. The 1-(2-nitrophenyl)ethyl moiety is converted to a 2-nitrosoacetophenone derivative[85, 87]. The general photochemical reaction scheme of an o-nitrobenzyl compound is shown in Figure 1-6.

![Figure 1-6 - O-Nitrobenzyl photochemistry[81, 89]](image)

### 1.5.3 Light Sources

Typical light sources for uncaging emit UV radiation to excite photolysis of photocleavable compounds. Flash lamps, lasers, transilluminators, and laser microscopes with UV emissions in the range of 300-360nm are often used for uncaging. Lasers with narrow excitations lines or broad bandwidth light sources equipped with filters to select for desired UV-range wavelengths are appropriate sources. Two-photon excitation sources are also of recent interest, where long wavelength light sources replace UV-light sources, and the virtually simultaneous absorption of two low energy photons[69] vs. one high energy photon produces excitation of the photochemical compound. The electronic properties of SNPs may offer the
potential of uncaging at higher wavelengths (visible to IR) due to SPR-enhanced excitation[90], which is of interest due to increased tissue penetration of longer wavelength light, and decreased probability of damage to biological components caused by high frequency (ultraviolet) electromagnetic energy.

1.6 Antisense Technology and Therapy

Antisense technology is the subject of much interest in the field of gene therapy, as a means to study gene function and to effect gene knockdown in the treatment of genetic disorders, cancers, and viral infections[91]. Antisense therapy functions on the basis of nucleic-acid based molecules whose sequence can be tailored to target the knockdown of specific gene products. This technology has been used control viral gene products and gene products associated with various cancers and autoimmune diseases, including notably Crohn’s Disease[92] for which the surface protein product ICAM1 is targeted by the antisense drug ISIS 2302 (ISIS Pharmaceuticals). Antisense ‘drugs’ consist of ssDNA, dsDNA, or RNA molecules which work at the mRNA or genomic level, preventing splicing, transcription, or translation, via steric hindrance or actual degradation of the targeted nucleic acid substrate. Antisense molecules take on several forms, including antisense DNA/RNA oligonucleotides, ribozymes, and small interfering RNAs (siRNAs), the latter being the most newly discovered form of antisense which is naturally found in many higher organisms.

1.6.1 Mode of Action

The concept underlying antisense technology is Watson-Crick base pairing[93] between a delivered nucleic-acid based molecule and its complementary intracellular target. By hybridizing to a complementary target, antisense oligonucleotides (DNA or RNA) or small interfering RNAs (siRNAs) inhibit either transcription or translation, and thus production of a specific protein
product. Antisense oligonucleotides function in one of two primary pathways: 1) enzymatic degradation of target mRNAs via RNase H 2) steric hindrance of translation, transcription, or splicing. Regardless of the mode of action, the end result of antisense therapy is the inhibition of a protein product encoded by the affected target nucleic acid. The RNase H pathway has been considered a gold standard in antisense technology, due to the fact that the antisense oligonucleotides involved are left intact and functional following target mRNA degradation, and are thus able to effect the degradation of many target mRNAs[94]. The lifetime of the antisense oligonucleotide in this case is the primary limitation of therapeutic effectiveness. Non-specific side effects of the therapeutic molecule as well as its intracellular localization may also limit the effectiveness of any particular antisense oligonucleotide once delivered to a cellular target.

RNase H is a ubiquitous enzyme, located in the cellular cytoplasm as well as in the nucleus, and is specific for RNA/DNA duplexes. Modifications on the sugar moieties of antisense oligonucleotides typically inhibit the action of RNase H[91], and the phosphorothioate oligo, in which a sulfur replaces the non-bridging oxygen of the phosphate backbone, is one of the few chemically modified antisense molecules which will still activate the RNase H pathway. However, even phosphorothioates have disadvantages compared to other antisense oligos, including non-specific interactions with proteins and decreased binding affinity for complementary nucleic acids when compared to traditional phosphodiester oligonucleotides. Studies have also been conducted showing attachment of phosphorothioate modified oligonucleotides to metal (gold) nanoparticles in studies on particle-particle linear connection/aggregation[95]. Alternative antisense oligonucleotides have been devised which bind tightly and specifically to target substrates, and which do not non-specifically bind to proteins or other macromolecules[93]. These ‘second generation’ oligonucleotides typically
function via inhibition of transcription or translation machinery once they are hybridized to
target DNA or mRNA substrates.

1.6.2 Antisense Oligonucleotides

Antisense oligonucleotides are short nucleic acids (10-30 base pairs long) [91] which interact with complementary mRNAs or duplex DNAs within the nucleus to prevent production of a specific protein product. This inhibition of protein product production at the transcription, splicing, or translation level is the hallmark of antisense technology, which may be contrasted to the activity of other drugs which may prevent the function of proteins or other biomolecule targets already present at the time of drug delivery. Antisense oligonucleotides prevent transcription or translation of substrate nucleic acids in a sequence-specific manner, and are often directed to the 5’ initiation sequences or 5’ UTRs (untranslated regions) when the mode of action is steric hindrance [65], or almost any available binding site on an mRNA when the mode of action is degradation. It is interesting to note that initiation sequences and UTRs are often available for binding due to the fact that these sites are generally accessible to proteins such as transcription factors and ribosomes [65].

A major problem with traditional (phosphodiester) antisense oligos is their susceptibility to degradation in the physiological environment [96]. The half-life of phosphodiester oligos, i.e. the time before half the molecules are inactivated by degradation, is only on the order of minutes when suspended in serum, or blood plasma. Serum contains 3’→5’ exonuclease proteins, which are the primary agents of degradation for traditional, i.e. phosphodiester, antisense oligos [97]. Endonuclease enzymes are also responsible, to a somewhat lesser extent, for oligonucleotide degradation in the intracellular environment. Thus, normal phosphodiester oligos have very limited use in antisense therapy, and have been replaced with chemically modified
oligonucleotides[93] (Figure 1-7) which resist degradation in a physiological environment. An important alternative to further chemical modifications of antisense oligonucleotides in expanding the utility and impact of antisense technology is the use of novel delivery routes[98] and drug carriers which may enhance function and prevent/delay the enzymatic degradation of associated nucleic acids. Significant effort is being dedicated to the discovery and synthesis of novel nanoparticulate carriers[65, 99] that will both protect and efficiently ‘shuttle’ drugs such as antisense oligonucleotides into cells[100]. Enhancement of antisense delivery and targeted release based on nanoparticle carriers is a primary subject of the studies herein.

The criteria of an ‘efficient’ antisense oligonucleotide (or AS-ODN) have been enumerated as follows[91]: sequence-specific target recognition, high affinity for target DNA or RNA substrates, resistance to nuclease activity, sufficient plasma half-life, minimal ‘off-target’ or non-sequence-specific effects, and in ideal cases activation of RNase H, which effects degradation of target RNAs. There have been many developments in antisense oligonucleotide chemistry, and many chemically modified oligonucleotides are now available which have longer half-lives in serum and resist nuclease degradation within the intracellular development. Phosphorothioate oligos, which have a modified phosphate backbone wherein a sulfur replaces the non-bridging oxygen, have a half-life in serum on the order of hours[100]. Phosphorothioate oligos, which are among the most extensively studied and practically applied antisense oligonucleotides[98], prevent mRNA translation via the RNase H degradation pathway. The use of this pathway for degradation of target nucleic acids, as well as the enhanced stability of phosphorothioates, creates the appeal of this particular antisense molecule. Other chemically altered oligonucleotides with modifications to the phosphate backbone, sugar moieties, or nucleotide bases [93, 98] have been employed to minimize degradation or off-target effects of
antisense drugs while preserving their specific hybridization to target DNAs/RNAs. Most of these modifications produce antisense molecules which function via steric inhibition of translation, splicing, or transcription of target nucleic acids. Antisense RNAs have also been chemically modified, via replacement of the ribose sugar 2’ oxygen with a 2’ O-methyl group or a 2’-florine, to resist enzymatic degradation and thus to have longer functional lifetimes in physiological environments.

Figure 1-7 - Chemically modified antisense oligonucleotides (Dias & Stein 2002)[93]
1.6.3 RNAi

RNA interference, or RNAi, is an emerging technology which involves the delivery of double-stranded siRNAs (small interfering RNAs) which activate intrinsic pathways leading to the enzymatic degradation of targeted (complementary) mRNAs[96, 101]. These pathways traditionally involve the enzyme complexes known as DICER and RISC, the latter of which functions in the unwinding of small double-stranded RNAs and in the cleavage of complementary RNAs[91]. RNAi, which is mediated by ssRNAs or small dsRNAs delivered to targeted cells, is related to endogenous microRNA pathways[91], which serve to naturally regulate gene expression in higher organisms including humans. Although extensive research is being conducted in the therapeutic utility of siRNAs, antisense oligonucleotides remain a primary tool for gene therapy due to greater flexibilities in the chemistry of these compounds, lower synthesis costs, high stability in most cases, and ease of handling and intracellular delivery[96].

1.7 Antisense Macromolecule Intracellular Delivery

The effective delivery of antisense oligonucleotides to the intracellular environment is an essential facet of antisense gene therapy. An effective delivery agent will ideally provide targeted (cell-, tissue-, organ-specific) delivery, enhance intracellular penetration, protect the antisense molecule from degradation, and prevent entrapment in endosomal/lysosomal compartments, while remaining minimally cytotoxic[100]. A further ideal in antisense delivery is specific targeting of therapeutics to particular tissues/cell types, as well as spatiotemporally controlled activation (‘uncaging’) or release from delivery vehicles[99]. ‘Drug-flooding’ is no longer a valid method of achieving therapeutic concentrations of drugs at target locations[68], thus we see increased importance of designing ‘smart’ delivery systems[68] for the more safe and efficient
delivery of antisense therapeutics. The manipulation of antisense oligonucleotides via chemical modifications may reach functional limitations, at which point vehicles for enhanced delivery, biodistribution, and controlled function of these therapeutics gain considerable interest in antisense technology. The limitations of antisense drugs which may be overcome by novel delivery vehicles include low permeability of the plasma membrane to naked oligonucleotides, difficulty of attaining therapeutic concentrations in target cells/tissues, and the tendency of foreign nucleic acids to be sequestered and degraded in endosomal/lysosomal intracellular compartments[100].

1.7.1 Overview of Delivery Methods

An entire field of research has been devoted to the development of methods for gene delivery to and transfection of cellular targets for the purpose of gene therapy. These methods may typically be classified according to their delivery approach: mechanical, electrical (electroporation), and/or chemical[102]. Many non-viral chemical approaches for nucleic acid delivery have been and continue to be the subject of extensive research. The efficacy of virus-mediated delivery has been a functional ideal in gene therapy, due to the natural ability of virus particles to traverse the cell membrane and efficiently insert their genome into host cells. However, due to the safety issues associated with use of viral carriers, non-viral carriers are a more viable option for enhanced antisense drug delivery. The intrinsically low permeability of the cell membrane to negatively charged nucleic acids has been surmounted by the use of cationic biomolecules, including synthetic lipids, polymers, proteins, and dendrimers, as non-viral delivery agents [63, 65, 103-104]. Cationic lipids and polymers are typically recognized as some of the most successful chemical delivery agents of therapeutic nucleic acids to date[63, 98]. The polar heads of cationic lipids interact electrostatically with DNA, and complex with the
DNA to form liposomes which can naturally cross the hydrophobic lipid bilayer of the cell membrane[67]. Cholesterol and ‘helper lipids’ such as DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) can aid membrane fusion[104] of the lipoplexes and enhance intracellular trafficking, respectively. Cationic polymers have also been successful in the efficient delivery of electrostatically complexed antisense nucleic acids. High buffering-capacity polymers, notably the cationic polymer PEI (polyethylenimine), have been used in order to help charge-associated oligonucleotides to escape from endosomal compartments via the proton sponge effect [105-106]; in essence the buffering capacity of PEI causes continual pumping of ions into the early lysosomal compartment, to the point at which the vesicle ruptures due to osmotic pressure, releasing the in-dwelling antisense molecules. PEI has been combined with other delivery vehicles [66, 106] in order to increase their effectiveness in the delivery of antisense molecules to the cytoplasm of target cells.

Although lipid and polymer transfection agents by themselves can be useful in loading large amounts of DNA into subject cells, they still suffer from limitations, including limited controllability, limited conferred stability onto electrostatically complexed nucleic acids, and a level of toxicity in most cases[50]. An exciting potential in drug delivery systems arises not from the use of these transfection components alone, but from the possibility of integrating these components with novel nanoparticles carriers to enhance cell delivery add create controllable and specifically targeted drug delivery vehicles. Lipid and polymer nanohybrid systems have displayed higher drug stability and transfection efficiency than non-particulate materials[63, 66, 106-107].
1.7.2 Nanoparticle Delivery of Antisense Drugs

Metallic nanoparticles offer the potential advantages of their electronic and optical properties along with the highest standards of antisense drug delivery vehicles. The advantages of metallic nanoparticles as delivery vehicles for antisense oligonucleotides include ease of nanoparticle synthesis, tailorable size and morphology properties, ease of surface ligand functionalization, presence of a solid surface for high density loading, enhanced stability and lifetime of bound nucleic acids[5], enhanced cellular uptake[11] with high loading densities, and potential of surface-enhanced fluorescence (detection) and photochemical processes. Metallic nanoparticles, almost exclusively gold nanomaterials, have been employed as novel antisense therapeutic vehicles when functionalized with antisense DNA[4, 11, 13] or siRNA[12, 108-110]. According to a recent review article in Science, the three biggest problems with RNAi therapeutics remains “delivery, delivery, delivery.”[111] While lipid and polymer-based particles and nanostructure carriers are currently the most widely used and viable delivery agents for antisense therapeutics, metallic nanoparticles provide the efficient delivery offered by these, while further providing ease of fabrication of multicomponent systems and enhanced control over intracellular release. Metallic nanoparticles may be functionalized with mixed monolayers for the further tailoring and enhancement of uptake, delivery, and release of antisense therapeutics. PEGylated siRNA gold nanoparticles have been reported with enhanced cell uptake and pronounced RNAi activity[108]. Novel multicomponent nanoparticulate systems for antisense delivery have been devised which incorporate uptake-enhancing and endosome-escaping polymers with metal nanoparticles serving as solid support delivery systems for nucleic acids [66, 110].
1.7.3 Silver Nanoparticles: Nontoxic Delivery Vehicles

In order for silver nanoparticles to be used in therapeutic delivery applications, the effects of SNPs on cell health must be studied, and shown to have no adverse affects at therapeutically applicable concentrations. The focus of nanosilver materials in medicine has been on their multilevel anti-microbial activity[112], with applications in wound treatment/healing[112-113], nanosilver-based dressings, surgical sutures, and surgical implants. Recently, studies on the use of SNPs in therapeutic applications, for example drug delivery, have met with concerns about the toxicity and negative effects of silver nanomaterials.

Concerns of the impact of silver nanoparticles on cell health include decrease of mitochondrial function and induction of apoptosis, the mitochondria being a sensitive target of cytotoxicity of SNPs[114]. While the mechanisms of silver nanoparticle toxicity are not well characterized, they may involve surface binding (depletion) of thiol-containing proteins, including glutathione and key component enzymes of the cell’s antioxidant defense mechanism, which leads to increased reactive oxygen species (ROS) generation, oxidative damage[114], and apoptosis, a complex programmed cell death pathway. Particle surface electrostatic interactions with charged biomolecules might also attribute to the toxic properties often observed for ‘naked’ silver nanoparticles at high concentrations[115]. While the negative effects of SNPs are of concern in therapeutic applications, especially the localization of SNPs in the mitochondria and generation of oxidative stress, current studies reveal that upon SNP uptake cellular antioxidant systems are triggered and their activity increased, preventing extensive oxidative damage[112]. Recent studies have also shown that primary cells have much higher SNP tolerance than concentrations often used for antimicrobial effectiveness[116]. Efforts to improve the biocompatibility of metallic nanoparticles include functionalizing the particles with
biocompatible biomolecules, i.e. poly(ethylene)glycol (PEG) groups, lipids, polymers, small peptides, etc. The surface modification of silver nanoparticles with thiol-modified biomolecules has been shown to improve silver biocompatibility and intracellular uptake[115]. In the cited study, phospholipid-protected silver nanoparticles were delivered into 3T3 fibroblast cells and platelet cells with minimal toxic effects. Thus silver nanoparticles may hold more potential in therapeutic applications than previously considered, and studies for the development on SNP-based antisense delivery platforms are justified. The effects on non-functionalized vs. nucleic-acid functionalized SNPs on cell health will be evaluated in studies proposed herein.

1.8 Model System for Antisense Therapy Evaluation

Model genetic systems are often useful for the analysis of the applicability of newly designed antisense therapeutics. Once a new delivery platform has been designed and characterized in vitro, analysis of performance must be carried out in the context of a living biological system, ex vivo/in vivo. Model systems may consist of transgenic cell lines induced to express specific protein products or markers, for example GFP (green fluorescent protein)[13], in high quantities for ease of antisense activity analysis of delivered therapeutics. Gene expression and silencing may be monitored by use of a model system when can be induced at a specific point in time, even after drug delivery, to begin expressing a targeted gene product.

The model system which will we will use is based upon the expression and production of intracellular adhesion molecule-1 (ICAM-1), a cell-surface transmembrane glycoprotein constitutively expressed by leukocytes and endothelial cells[117]. ICAM-1(CD54) mRNA expression and protein production can be upregulated in a dose-dependent manner by inflammatory mediators such as the cytokine interferon gamma (INF-γ)[117-118]. The ICAM-1 protein can easily detected by anti-human CD54 fluorophore-labeled monoclonal antibody, a
detection mechanism which serves as a validation of the expression level and knockdown activity of antisense oligonucleotides specific for ICAM mRNA. This makes ICAM upregulation as a molecular method for antisense activity analysis especially attractive, as live-cell surface protein antibody labeling is a valid indication of gene expression levels. A well known antisense oligonucleotide targeting the ICAM-1 mRNA (ISIS 2302, ISIS Pharmaceuticals Inc., Carlsbad, CA), developed for the treatment of Crohn’s Disease[98], will be employed to investigate the activity of caged/uncaged SNP-antisense conjugates in our ex vivo studies.

1.9 Significance

Targeted delivery and controlled release of therapeutics in vivo, including antisense nucleic acids, are important goals of modern biotechnology and medicine[2]. Biomolecule functionalized metal nanoparticles with photo-responsive moieties[68] hold much potential as stable drug carriers with unique optical and photo-activated properties. Silver, as one of best surface-enhancing substrates, is a prime choice for studies on the potential of metal nanohybrids as novel ‘caging’ compounds with metal-enhanced photo-release of cargo therapeutics.

Studies focusing on the advancement of delivery agents and targeting/release methods have risen to the top of the list of goals in nanomedicine and antisense technology[16]. While antisense therapeutics, as drugs which can be tailored to target any gene or gene regulator for which we know the sequence, are potential ‘superdrugs’ for gene therapy, novel delivery technologies are required to enhance their efficacy, increase their physiological stability, and minimize side effects in vivo[91]. Cell uptake and intracellular trafficking of delivered therapeutics, such as antisense molecules, is a complex process, and may require multicomponent systems with diverse functions for optimal delivery. Tekmira Pharmaceuticals’ SNALP (stable nucleic acid-lipid particle) technology, which has been used in Phase 1 clinical
trials, incorporates “four distinct lipids, which play distinct roles in the pharmacokinetics and pharmacodynamics of the product.”[111] Metal nanoparticles functionalized with biomolecules are prime targets for the development of multicomponent systems, incorporating diverse levels of function within the physiological environment. Metal nanoparticles provide a solid support for attachment of both the cargo therapeutic and delivery-enhancing agents (lipids, polymers, peptides, etc.), unique electronic and optical properties with applicability to enhanced spatiotemporally controlled release and activation, tailorable surface distribution of ligands for either enhanced or decreased (caged compounds) availability of surface-bound therapeutics for binding to physiological targets, and many other novel delivery properties.

To date, most nanoparticle-based drug delivery has taken advantage of relatively inert gold nanoparticles and other noble metal nanomaterials, with the exception of silver. This is due to the difficulty of silver synthesis and functionalization compared to that of gold, lesser stability when functionalized according to popular salt-aging techniques, and concerns about silver toxicity. However, recent improvements in silver nanoparticle biocompatibility via surface modification and the exceptional optical and surface-enhancement properties of silver nanoparticles has led to interest in developing an SNP-based antisense drug delivery platform for photo-activated gene silencing, which is the subject of the herein proposed studies.

1.10 Potential Difficulties and Alternative Methods

Difficulties which have been and may still be encountered in this project are diverse, including maintaining colloid stability during functionalization and purification, obtaining photorelease of particle-bound oligonucleotides while preventing false-negative results due to non-specific oligonucleotide interactions with the metal surface, and both foreseen and unforeseen difficulties with achieving efficient cell uptake with no adverse effects on cell health,
diffuse intracellular distribution, and detection of SNP-oligonucleotide conjugate release properties in vivo.

1.11 References


Chapter 2. Silver Nanoscale Antisense Drug Delivery System for Photoactivated Gene Silencing

2.1 Introduction

Targeted delivery and controlled release of antisense therapeutics in vivo are essential aspects of an ‘ideal’ delivery vehicle[100]. In this study we have undertaken the synthesis and in vitro/intracellular characterization of silver nanoparticle (SNP) photolabile nucleic acid conjugates, with the aim of developing a silver nanoparticle-based platform for therapeutic oligonucleotide delivery and photo-activated gene silencing. Nanostructures, which are amenable to molecular detection, intracellular diagnostics, and drug delivery applications due to their submicroscopic dimensions (<1 um) and the unique electronic and photonic properties which emerge at these dimensions, have emerged been increasingly utilized within the last decade[1]. The vast majority of nanoscale delivery platforms to date are polymeric in makeup. However, a focus on metallic nanoparticles offer benefits for drug delivery applications due to their tunable size, shape, and functionality attributes; relative ease of bulk synthesis via ‘wet chemistry’ techniques[2]; large surface area-to-volume ratio; simple covalent coupling chemistries to metallic surfaces [3-4], enhanced stability of surface-bound macromolecules including nucleic acids[4-5], and the exploitation of unique intrinsic photophysical properties of small noble metal particles[17]. Here the photophysical properties of silver nanoparticles are harnessed for both enhanced detection and spatiotemporal control over release of surface-bound therapeutics.

Antisense therapeutics, powerful nucleic acid drugs tailored to target any gene product for which the genomic, mRNA, or regulation sequence is known, are potential ‘superdrugs’ for gene therapy[91]. However, improved delivery technologies are required to enhance their efficacy, increase their physiological stability, and minimize side effects in living systems. Cell
uptake and intracellular trafficking of delivered antisense therapeutics is a complex process, and may require multicomponent systems with diverse functions for optimal delivery. Antisense-nanoparticle composites are prime targets for the development of multicomponent systems, incorporating diverse levels of function within a single drug delivery platform.

Here we report the synthesis of UV-photo-activated silver nano-carriers of antisense therapeutics which, when compared to traditional solution-phase nucleic acids, exhibit enhanced stability, decreased susceptibility to enzymatic degradation, enhanced cellular uptake, and enhanced cellular distribution and activity upon photo-release. To date, most nanoparticle-based drug delivery has utilized relatively inert gold[11-13] and other metallic and non-metallic nanomaterials[50, 119]. Silver has not been applied in such applications, likely due to the difficulty of silver synthesis and functionalization compared to that of gold, lesser stability when functionalized according to popular salt-aging techniques, and concerns about silver toxicity. However, recent improvements in silver nanoparticle biocompatibility via surface modification, as well as the exceptional optical[22, 42] and localized surface plasmon resonance (LSPR) enhancement[29] properties of silver nanomaterials have led to its suitability for this application. Localized surface plasmons (LSPs), excitations of the conduction-band electrons of sub-wavelength conductive nanoparticles[18], create a condensing effect of incident light at particle surfaces, which in turn produces large local enhancements of E-fields. These enhancements form the basis of surface-enhanced spectroscopy and photochemistry techniques[9]. The unique light-scattering and field-enhancement properties of silver nanoparticles, where the scattering cross section of a silver particle is about 10 times greater than that of a gold particle of the same size[22], have led to increased interest in their use as sensors[34, 49], biological labels[22], and substrates for surface-enhanced absorption,
fluorescence[120], and photochemistry[17, 121]. Our SNP-based platform for antisense drug delivery takes advantage of the unique photo-physical properties of silver nanoparticles for fluorescence confirmation of surface functionalization, cell uptake, and light-triggered activation of therapeutic SNP-oligo conjugates.

2.2 Experimental Approach

The fabrication of silver nanoparticle (SNP)-based antisense drug delivery vehicles was initiated with synthesis of raw SNP substrates and functionalization of substrate surfaces with oligonucleotide ligands, and completed with full solution and in vitro cell culture characterization of oligonucleotide nanocomposites. A citrate-reduction SNP synthesis method was chosen due to ease of bulk synthesis within the desired 40-80nm diameter size range and simple replacement of the citrate capping moiety (citrate) with thiol-modified nucleic acids. A Tris-buffer based salt-aging process[56] was used adapted from a Lee and Meisel method to develop stable SNP-conjugates functionalized with fluorophore-labeled thiol end-modified DNA oligonucleotides. These oligonucleotides were synthesized with an internal nitrobenzyl photocleavable linker (IDT, Coralville, IA) which when exposed to UV-wavelength light, initiates disengagement of a functional oligonucleotide from the protected nanohybrid surface.

Physiochemical confirmation of the synthesized SNP-oligonucleotide conjugates was accomplished via UV-Visible spectroscopy, transmission electron microscopy (TEM), and fluorescence-based surface coverage analysis techniques. These techniques served to characterize and quantify ligand attachment, stability, and surface properties of the SNP-oligonucleotide conjugates. Functional properties of the SNP-oligo conjugates were assessed via hybridization and nuclease digestion assays, the goal of these studies being to prove enhanced stability and reduced availability of particle-immobilized oligonucleotides for hybridization prior
to photo-activation. Photoreactivity of SNP-bound oligonucleotides possessing internal photocleavable (PC) nitrobenzyl linkers were characterized and in future work will be compared to that of identical oligonucleotides in solution (non-immobilized) via fluorescence-based analyses and HPLC.

Cell culture studies were utilized for confirmation of SNP-oligo conjugate intracellular delivery, ligand photo-release, sub-cellular localization, and photo-activated antisense activity, which were assayed via time-lapse fluorescent confocal imaging and TEM. The antisense activity of developed SNP-oligo conjugates was demonstrated via ICAM1 (Intracellular Adhesion Molecule-1) protein expression knockdown in HeLa cells.

2.3 Study Objectives and Work Plan

The approaches for our work are classified into the following experimental objectives: silver nanoparticle (SNP) synthesis and characterization; SNP conjugate synthesis via surface functionalization techniques; In solution qualitative, quantitative, and functional characterization of SNP conjugates; evaluation of SNP conjugate photochemistry; and finally in vitro cell culture studies of SNP conjugates as photo-activated antisense drug delivery vectors. We will utilize primarily optical-based techniques to accomplish our objectives, including TEM, absorbance and fluorescence spectroscopy, and confocal microscopy.

2.3.1 Reagents

2.3.1.1 SNP Synthesis and Functionalization

Silver nitrate (SigmaUltra >99%) and sodium citrate were purchased from Sigma Aldrich (St. Louis, MO). Other materials for SNP synthesis included PTFE coated stir-bars (VWR, West Chester, PA), 100ml glass syringes (KD scientific, Holliston, MA), and SNP synthesis-reserved glassware (Fisher Scientific, Pittsburgh, PA). Silver surfaces were functionalized with custom
thiol-modified oligonucleotides ordered from Integrated DNA Technologies (IDT, Coralville, IA). 2M Tris-NaCl buffer (pH 8.0) was made up from Biotechnology grade Tris (Amresco Inc, Solon, OH) and sodium chloride (Fischer Scientific, Pittsburgh, PA). Dithiothreitol (1M aqueous) purchased from Sigma (St. Louis, MO) was employed for DTT-mediated oligonucleotide removal from SNP surfaces.

2.3.2 Methods

The following flow chart is a representation of the methods employed in synthesis and full characterization of SNP-oligo conjugates:

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**Figure 2-1** – Flow diagram of experimental objectives and work plan
2.3.2.1 SNP Synthesis

Citrate-reduced silver colloid was prepared according to a modified Lee and Meisel method[43]. Silver nitrate (90mg) was suspended in distilled water (500mL), and heated rapidly to boiling (95-100°C) under continuous stirring. Upon boiling, a 1.0% solution of sodium citrate (10mL) was added dropwise with a syringe-pump automated flow-rate (0.5 mL/min), while solution color progression from pale yellow to dark green signified formation of silver colloid. The solution was allowed to boil for the duration of citrate addition plus 10 minutes following addition, to ensure complete reduction of silver nitrate. The colloid was allowed to cool to room temperature prior to characterization via visible absorption spectroscopy and transmission electron microscopy (TEM).

![Schematic of Fluor-Oligo functionalized silver nanoparticle](image)

**Figure 2-2** Schematic of Fluor-Oligo functionalized silver nanoparticle
2.3.2.2 Design of SNP-conjugate Oligonucleotides

The photo-labile oligonucleotides for conjugation to SNP surfaces were designed in such a manner as to optimize surface coverage, SNP-conjugate stability, and surface-enhancement of photochemical processes, including fluorescence detection and photolysis of the nitrophenylethyl (NPE) internal photocleavable linker (Figure 2-2). A 10mer thymine linker was incorporated into the 3’thiol-modified oligonucleotides in order to enhance particle surface coverage, due to the fact that thymine has been previously shown to have the least affinity for metal surfaces of the four available nucleotides[48], thus reducing non-specific oligo-metal interactions. Spacer thiols have also been shown to reduce the non-specific adsorption of nucleic acids onto metal particle surfaces[52] by competing for surface attachment sites, thus an alkane spacer thiol (mercaptopropanol) has been incorporated which adsorbs to the metal surface concurrently with the target oligonucleotide (Figure 2-2).

The particle-bound thiol-modified oligos have as their base the ISIS 2302 sequence, which is an established antisense sequence against ICAM-1 (CD54) [98]. This system will serve as a proof of concept in studies on SNP-mediated antisense drug delivery and controlled release. A simple thiol-modified ISIS 2302 sequence oligonucleotide (1a) was used as a control for immobilization of nucleic acid onto silver surfaces and characterization of surface coverage. A TYE(563) fluorophore-labeled oligonucleotide (1b) was designed for fluorescence detection of oligonucleotide in solution and attached to SNPs. The excitation/emission (549/563nm) properties and particle-fluor spacing (10-12nm based on a 30mer separating oligonucleotide sequence) were chosen to minimize overlap with intrinsic SNP fluorescent properties in the SPR region and quenching by the silver surface. Of the two oligonucleotides studied for photocleavage analysis, the photocleavable group in the TYE-NPE(1n)-oligo (2a) (Figure 2-3) is
spaced from the particle surface by a three carbon alkane thiol (ThiolMC3) plus one thymine nucleotide, while the photocleavable group in the \textit{NPE(10n)-oligo} (2b) (Figure 2-3) is spaced from the particle by a three carbon alkane thiol plus a thymine linker sequence of 10 nucleotides. The thiol linker (3-carbon alkane chain) is estimated to have a linear length <1nm, based on reported C-C, C-S, and S-Ag bond lengths[122], and the nitrobenzyl linker an estimated linear length slightly greater than 1nm based on traditional bond lengths. Oligonucleotide length is estimated based on a 0.3-0.35nm/base unit length[123]. This leads to particle-to-photolabile linker distances for the SNP-\textit{TYE-NPE(1n)-oligo} conjugates and SNP-\textit{NPE(10n)-oligo} conjugates of 2-3nm and 5-6nm, respectively. The different particle to photocleavable group spacing distances will serve to help evaluate distance-dependent influences of silver surface-plasmon resonance on the NPE photochemical reaction.

**Figure 2-3** - Design of current oligonucleotides for SNP functionalization. 1a) Thiol-modified oligonucleotide 2a) Fluorophore-labeled thiol-modified oligonucleotide with 1-thymine linker (\textit{TYE-NPE(1n)-oligo}). 2b) Non-labeled thiol-modified oligonucleotide with 10-thymine linker (\textit{NPE(10n)-oligo})

### 2.3.2.3 SNP Functionalization

Silver nanoparticles were functionalized via a modified ‘salt-aging’ technique. Custom thiol-modified TYE563\textsuperscript{TM}-labeled oligonucleotides (Integrated DNA Technologies, Inc) were mixed with silver colloid (50pM) at a oligo-to-particle stoichiometry of 5000:1, and left to incubate at 37°C under gentle rocking conditions to favor initial oligonucleotide. Following a 24hr incubation period, a 48hr Tris-buffer based salt-aging process was commenced during
which the SNP-oligo conjugate solution was first rapidly adjusted to 1% SDS and 25 mM phosphate buffer, and subsequently slowly (4x 10ul aliquot additions of a 1M Tris-NaCl buffer per 24hrs) adjusted to 80mM NaCl and 80mM Tris buffer (pH 8.0) concentration. Functionalized nanoparticles were purified via centrifugation (3 x 7000 RPM) and resuspended in a modified buffer (1% SDS, 50mM Tris-NaCl) prior to experimental analysis.

2.3.2.4 Naked and Functionalized SNP Characterization

For UV-Visible and fluorescence spectroscopic characterization, naked and oligonucleotide functionalized SNPs were diluted to appropriate concentrations in DI water. Diluted samples were analyzed for absorption properties in a wavelength range scan (200-700nm), and for single excitation (420nm and 520nm), multiple emission fluorescence properties using a PE LSB-50 spectrofluorimeter. Spectra were normalized to maximum SPR absorption and particle fluorescence conditions.

For TEM characterization, 5 ul of SNP solution and 5ul of SNP-Thiol-oligo(1a) conjugate solution were pipetted on Carbon/Copper 20-30 nm grids (EMS, Hatfield, PA), air dried and visualized with JEOL 100CX. MetaVue image analysis (Universal Imaging Corporation, West Chester, PA) was employed to determine particle counts and average particle size from TEM images.

2.3.2.5 DNA Coverage Quantification

DNA coverage on functionalized SNP-conjugates was quantified via fluorescence-based measurements and confirmed via gel electrophoresis of particle-released oligonucleotide samples. Following SNP-conjugate functionalization and purification via centrifugation (3x 7000RPM), 1% dithiothreitol (DTT) was added to SNP-conjugate solutions and allowed to shake at 37 °C for 10-15min. Following an additional purification step to remove DTT-released
oligonucleotide from silver colloid (1x 7000RPM), released oligonucleotide-supernatant was analyzed via fluorimetry with excitation/emission lines set at 549/563nm (TYE-fluorescence spectra, Integrated DNA Technologies, Coralville, IA) on a PE LSB-50 spectrofluorimeter. A TYE-oligo fluorescence calibration curve was used to indirectly quantify TYE-oligo coverage on SNP surfaces based on solution measurements (supplemental information Figure 2-14).

2.3.2.6 In Vitro Hybridization Activity

Hybridization activity studies were carried out following SNP functionalization and conjugate purification. A FAM-BHQ molecular beacon with a sequence complementary to the ISIS 2302 sequence of the oligonucleotide ligands was hybridized to both SNP-bound and SNP-released oligonucleotide samples. Hybridization conditions were 30min at 60 °C followed by 15min at 4°C. These conditions were chosen based on a T_M of 40.7 °C for the highest energy hairpin folding structure of the molecular beacon (IDT SciTools mFold, Coralville, IA). Releasing conditions included both UV-light exposure and 1% DTT treatment. Oligonucleotide and oligonucleotide-beacon duplex samples were purified and removed from silver nanoparticle surfaces prior to fluorescence analysis via fluorimetry with excitation/emission lines set at 549/563nm for TYE-fluorescence and 480/520nm for FAM-fluorescence. For SNP-bound samples, FAM-BHQ molecular beacon was added to 500ul of SNP-oligo conjugates at a final concentration of 0.6uM (.3 nmoles). Following hybridization, free molecular beacon was removed via centrifugation (1x 7000RPM), and oligonucleotide and duplex ligands were removed from particle surfaces via DTT treatment (1x 7000RPM) for fluorescence and gel analysis. For SNP-released samples, oligonucleotide was removed via DTT after which FAM-BHQ molecular beacon was added, hybridized, and purified from SNP surfaces (1x 7000RPM) similar to above. Fluorescence and gel analysis provided comparison of ssDNA vs. DNA-beacon
duplex in both SNP-bound and SNP-released cases. A solution hybridization control was run via gel electrophoresis (.15 nmoles FAM-BHQ molecular beacon, .05 nmoles TYE-oligo) to confirm gel results of experimental samples. All samples were desalted via spin columns (3kDa Amicon Ultra filters, Millipore, Billerica, MA) and dried via vacuum centrifuge to a final volume of <10ul for final gel preparation. Samples were analyzed via UV-gel electrophoresis and Sybr Green staining (Invitrogen Corporation, Carlsbad, CA).

**Figure 2-4** - Hybridization assay schematic. A) Quantification of hybridization of molecular beacon (MB) to TYE-NPE(1n)-oligo in particle-bound case. B) Quantification of hybridization of MB to TYE-oligo in DTT-released and photo-released cases.

### 2.3.2.7 In Vitro Protection from Nuclease Activity

Protection of SNP-bound oligonucleotides from nuclease activity was investigated via a DNase I digestion assay on free solution DNA and surface-bound DNA alike. 1ml of prepared SNP-TYE-oligo conjugate was purified via centrifugation (2x 7000RPM) and resuspended in 500ml of 1x DNase I NEB buffer (New England BioLabs, Ipswich, MA). 1.5ug of free solution TYE-labeled oligonucleotide was also suspended in 500ml of 1x DNase I NEB buffer prior to DNase I digestion. 4U of DNase I (stock concentration 2U/ml) was added to sample solutions
and incubated at 37°C for intervals of 5, 15, and 45min. Following digestion intervals, DNase stop buffer (.5M EDTA) was added to each sample to a final concentration of 5mM. SNP-TYE-oligo conjugates were purified via centrifugation (1x 7000RPM) followed by 1% DTT-mediated removal of DNA as described previously. Free solution and particle-bound DNA samples were desalted via spin columns (3kDa Amicon Ultra filters, Millipore, Billerica, MA) and dried via vacuum centrifuge to a final volume of <10ul. Samples were analyzed via gel electrophoresis and fluorescent-gel imaging (Typhoon 8600, GE Healthcare Life Sciences, Piscataway, NJ).

2.3.2.8 In Vitro Photo-release Efficiency

Proof of photolabile oligonucleotide release from particle surfaces was demonstrated via solution fluorescence and fluorescent gel electrophoresis mediated detection of TYE-labeled nucleic acid ligands. 1ml of prepared SNP-fluor oligo conjugates were loaded in a demountable quartz cuvette chamber (5mm path length, 49-Q-5, Starna Cells Inc, Atascadero, CA) and irradiated at 320nm by means of a UVP-Transilluminator and at 365nm by means of a GreenSpot system (American Ultraviolet, Lebanon, IN), which incorporates a 100–watt, pressurized mercury lamp with 5mm x 1000mm light guide, and produces a peak spectral output at 365nm (American Ultraviolet, Lebanon, IN). The lamp has a fluence of 206 mW/cm² with the short bandpass (1.5mm thick, 2.4mm diameter SWP–2502U–400, Lambda Research Optics, CA) and IR filters (818–ST–UV detector, Newport Corporation, Irvine, CA) in place. Released oligonucleotides were purified via centrifugation (1x 7000RPM) from SNP substrates in order to conduct fluorimetry and fluorescent gel electrophoresis (Typhoon 8600, Excitation laser: 532nm, Emission filter: 560LP) analysis. Extinction coefficient values for the internal NPE linker were calculated according to absorption values found by absorption spectroscopy of TYE-NPE(In)-oligonucleotide (2a) with an incorporated NPE photocleavable linker.
2.3.2.9 Cell Culture

HeLa cells (human epithelial carcinoma cells, American Type Culture Collection) were maintained in 25 cm² flasks (BD Falcon, Franklin Lakes, NJ) with 5 mL of Dulbecco’s Modified Eagle’s medium-reduced serum (DMEM-RS, Hyclone Thermo Scientific, Logan, UT) supplemented with 3% fetal bovine serum (FBS, Hyclone Thermo Scientific, Logan, UT) and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Opti-MEM® (OM, Invitrogen Corporation, Carlsbad, CA) is a reduced protein non-phenol-red medium which was used for imaging purposes.

2.3.2.10 Confocal Microscopy Analysis of SNP-conjugate Delivery, Subcellular Localization, and Photo-release Profile

Confocal microscopy was used to monitor SNP-oligo conjugate intracellular delivery, trafficking, and photo-release. For imaging purposes, HeLa cells were seeded in 1.0 Borosilicate coverglass Lab-Tek™ 8-well chambered slides (Thermo Fisher Scientific, Rochester, NY). Final cell media volume in each chamber well was 400ul. Cells were allowed to incubate for 24hrs in DMEM+FBS media at 37°C prior to SNP-oligo conjugate treatment. 5ul of 1x naked SNPs and 5ul of concentrated 5x SNP-oligo conjugates (50pM, ≈1-2ug oligo) were added directly to culture 8-wells. Treated cells were incubated at 37°C in a CO₂ incubator for 16 hrs following addition prior to live confocal imaging. For subcellular localization of SNPs vs. SNP-thiol-oligo conjugates, treated cells were stained with LysoTracker (Invitrogen Corporation, Carlsbad, CA) for 1hr at 50nm staining concentration. For photo-release profile studies, cells were flashed for 3min @365nm (18 J/cm²) 2hrs prior to imaging (16hrs post SNP-Fluor-oligo conjugate delivery). All samples were resuspended in Opti-MEM® prior to imaging. The Leica TCS SP2 spectral confocal & multiphoton system used consists of a Leica DM IRE2 inverted microscope with a galvo-Z stage, equipped with Ar/Kr, He/Ne green, and He/Ne red lasers. Of the provided
laser lines, excitation lasers at 488, 543, and 633nm were used in imaging experiments, concurrently with tuned emission wavelength windows. Reflectance mode images of silver nanoparticles were acquired by centering the emission wavelength directly on the 488nm excitation line. Images were analyzed using Leica Confocal Software (LCS) Lite (Leica Microsystems Heidelberg GmbH).

2.3.2.11 Antisense Activity of SNP-oligo Conjugates

Antisense activity of SNP-oligo conjugates against plasma membrane surface protein ICAM1 in HeLa cells was monitored via confocal microscopy. HeLa cells were seeded as described above. ICAM1 antisense oligonucleotide transfection was carried out 16hrs prior to experimental analysis via TurboFect™ cationic polymer for naked DNA delivery, and SNP-oligo conjugates for nanoparticle-bound DNA delivery. TurboFect-oligonucleotide polypelex samples were prepared by mixing 1.5ug of TYE-NPE(1n)-oligonucleotide or phosphorothioate ISIS2302 oligonucleotide with 0.8ul of TurboFect stock reagent in 100ul OM for 15min at room temperature and then added directly to culture 8-wells in a 1:5 volumetric ratio. SNP-Fluor oligo conjugates were added to culture wells as described previously. ICAM1 was upregulated in HeLa cell samples with recombinant human interferon-gamma (INF-γ, 16.9 KDa, eBioscience®, San Diego CA) by adding 10ug/ml to culture wells 12hrs prior to Anti-human CD54-APC (Allophycocyanin) conjugated antibody staining (Mouse monoclonal to ICAM1, AbCam®, Cambridge, MA) and confocal imaging. Photo-release of antisense ISIS2302 from SNP-conjugate surfaces was effected by flashing @ 365nm 6hrs post-INF-γ treatment and 6hrs pre-imaging. Flashing doses were as follows: 15 J/cm² (low flash), and 25 J/cm² (high flash). Cells were rinsed with and stained in Opti-MEM® with 2%BSA for 1hr with 10ul CD54-APC antibody stock and finally resuspended in Opti-MEM® for imaging purposes.
2.4 Results

2.4.1 SNP Synthesis

Synthesized silver colloids were characterized via optical techniques including UV-Vis spectroscopy and transmission electron microscopy (TEM). Based on TEM images, the average particle diameter was calculated to be 60nm±20nm. Particle size is dependent upon the ratio of reducing agent to silver nitrate in the sodium-citrate reduction synthesis reaction, and was observed to be slightly variable from synthesis to synthesis. A broad optical spectrum centered around 420nm is expected for citrate-reduced silver particles in this size-range, the shoulder around 345nm being attributable to multipole resonance modes of the SNPs[22], absorbances above 550nm attributable the aggregates or larger rod-like structures[124]. Using a reported extinction coefficient value for silver nanoparticles of approximately 60nm[22, 42] (6.75 x 10¹⁰ M⁻¹ cm⁻¹), the yield concentration of 60nm diameter citrate-reduced silver colloid was determined to be 50±10pM according to maximum SPR absorption. This may be compared to a theoretical yield of 72pM, based on complete reduction of silver colloid synthesized above. Once synthesized, the Ag colloid was stable for 2-3 weeks in dark conditions at 4°C.

2.4.2 Naked and Functionalized SNP Characterization

UV-Visible spectroscopy serves to probe changes in the SPR spectra of silver nanoparticles occurring with absorption of ligands at the particle surface, i.e. change in the dielectric environment [17, 29, 125]. Changes in the local dielectric environment around SNPs affect SPR spectra due to Mie Theory, which describes the extinction properties of small metal particles[19]. Salt aging functionalization techniques and absorption of ligands to the SNP surface results in damping of the SPR peak intensity and shifting of the peak resonance frequency, with an increase in the dielectric constant of the local environment typically causing a
red shift in the SPR absorption band [29]. This phenomenon has been observed in our synthesized SNP-oligo conjugates, with slight red-shifting of the SNP resonance peak providing evidence of ligand attachment to particle surfaces (Figure 2-5 a). SNP-conjugates also underwent a 4-5x decrease in concentration during functionalization as monitored by decrease in SPR absorbance intensity, but remained stable during subsequent treatments including centrifugation, resuspension, and UV-light exposure.

TEM analysis of non-functionalized and thiol-modified oligonucleotide functionalized SNPs (Figure 2-5 b&c) reveal an average ‘naked’ SNP diameter of 68nm ± 20nm, and an average SNP-Thiol-oligo conjugate diameter of 74nm ± 20nm, based on particle counts (n = 50) and image analysis. Variegated SNP morphologies with a predominance of moderately sized rough spherical or spheroidal particles are characteristic of citrate ion reduction methods [126]. SNP-thiol oligo conjugates were observed to be surrounded with light interfering regions attributed to adsorbed oligonucleotides and associated salts (Figure 2-5 panel c) inset). TEM sample preparation, which involves dehydration of SNP and SNP-conjugates, and heating during imaging may affect surface tethered oligonucleotide layers.

Fluorescence spectroscopy emission scans of naked SNPs vs. purified SNP-TYE-oligo conjugates (Figure 2-6) reveal the broad fluorescence properties of silver nanoparticles and conjugates when excited at 420nm. An excitation line at 420nm was chosen due to proximity to the maximum SPR absorbance peak of 60nm silver particles. Fluorescence emission spectra are also shown with an excitation line at 520nm, which provide evidence of TYE563-labeled oligo attachment to SNP surfaces.
Figure 2-5 – Absorbance spectroscopy and TEM of SNPs and SNP-oligo conjugates. a) Normalized absorbance spectra for non-functionalized silver nanoparticles (SNPs) and thiol-modified oligonucleotide functionalized silver nanoparticles. b) TEM images of naked (non-functionalized silver nanoparticles and c) oligonucleotide-functionalized silver nanoparticles.

Figure 2-6 – Fluorescence emission scans of SNPs vs. SNP-TYE-oligo conjugates at 420nm and 520nm excitation lines. Black-dotted lines represent 420nm excited spectra, red-dotted lines represent 520nm excited spectra. Inset pictures demonstrate naked vs. oligo functionalized silver nanoparticles.
2.4.3 DNA Coverage Quantification

Oligonucleotide coverage on the SNP-conjugate surface is an important aspect in terms of conjugate stability, efficiency of therapeutic delivery, and availability of attached oligonucleotides for hybridization[52]. Oligonucleotide loading density on nanoparticle surfaces is impacted by final salt concentration, oligonucleotide sequence, presence of spacers in mixed monolayers, and particle size/radius of curvature[57, 127]. A fluorescence-based method for quantifying oligonucleotide coverage on SNP surfaces, similar to that used by Demers el al[55], has revealed an SNP-Fluor oligo coverage density of 8.85(±2) x10^{12} molecules/cm², with an oligonucleotide footprint[57] of 11.3±2 nm². These values, calculated for 60nm diameter SNPs and a surface coverage ratio of 1000:1 oligonucleotide ligands per particle, are comparable to or higher than those reported previously for DNA-functionalized gold nanoparticles of similar dimensions (reported coverage and footprint for 60nm gold particles 7.8(±1)10^{12} oligos/cm² and 13±2 nm², respectively) using PBS-buffer based salt-aging functionalization processes[57]. Complete DTT-mediated oligonucleotide removal[55] was confirmed via absorption spectroscopy analysis of colloid solutions, where DTT-mediated ligand removal promotes aggregation of silver nanoparticles and red-shifting of SPR spectra following ligand desorption.

2.4.4 In Vitro Hybridization Activity

In vitro hybridization studies reveal a caged phenomenon of SNP-tethered oligonucleotides. Oligos packed on the SNP surface are inactivated from their normal function, i.e. hybridization to a complementary target, until release and detachment from the particle surface. Increases in molecular beacon fluorophore (5’ FITC) fluorescence and quenching of TYE-labeled oligonucleotide fluorescence due to proximity to molecular beacon quencher (3’ BHQ1) revealed an increase in availability of oligonucleotide ligand for hybridization to its
target sequence following chemical release from the SNP surface (Figure 2-7). Solution fluorescence measurements were taken in absence of silver nanoparticles, following chemical release of all ligand, due to interference of beacon emission and SNP emission at 520nm. Solution measurements were further confirmed via gel electrophoresis of particle-bound and DTT-released hybridized samples. Similar results were obtained showing an increase in hybridization following photo-release from SNP surfaces. The SNP in this case functions as a bulky caging group, inactivating the attached therapeutic nucleic acid until controlled, on-site photo-activation.

Figure 2-7 – Hybridization of particle-bound vs. released oligonucleotide ligands. a) Graph of solution fluorescence data for FAM-labeled molecular beacon (MB) and TYE563-labeled thiol-modified oligonucleotide. Sample 1 represents hybridization of a complementary MB to particle-bound oligonucleotide, whereas sample 2 represents hybridization of a complementary MB to a released oligonucleotide, as measured by open-beacon FAM fluorescence and TYE quenching by the beacon’s BHQ1. b) Gel electrophoresis run of samples 1&2 in duplicate.

2.4.5 In Vitro Protection from Nuclease Degradation

Results from a DNase I digestion assay confirm previously proven phenomena of nuclease resistance for gold nanoparticle-tethered nucleic acids[5]. SNP-tethered TYE563-
labeled oligonucleotides were effectively protected from nuclease action, as demonstrated by stability over time in the presence of DNase I for 0, 5, and 15min (Figure 2-8 bottom). Similarly treated TYE563-labeled oligonucleotide samples free in solution suffered significant nuclease degradation over the course of 15 min (Figure 2-8 top). TYE fluorescence measurements of aspirated solution following centrifugation of DNase I treated SNP-oligo conjugates also confirmed minimal nuclease degradation from nanoparticle surfaces (data not shown).

**Figure 2-8** – DNase I digestion assay of particle-bound vs. solution-phase oligonucleotides. a) Schematic of DNase I activity on solution-phase (top) and particle-bound (bottom) oligonucleotide. b) Gel electrophoresis run of nuclease digested solution-phase samples (top) and DTT-removed particle-bound samples (bottom) at 0, 5, and 15min nuclease treatment times.

### 2.4.6 In Vitro Photo-release and Photo-release Efficiency

In vitro studies confirmed the release of oligonucleotide ligands with internal NPE linkers from the surface of silver nanoparticles at photo-irradiation wavelengths between 302nm and 365nm. UV-Vis spectra were monitored throughout functionalization, UV-light exposure, and DTT mediated release of oligonucleotide ligands from SNP surfaces (Figure 2-9 a). Slight red
shifting in flamed sample may be due to minimal particle aggregation due to exposure of concentrated SNP-conjugate samples to high UV-light doses in order to achieve efficient photo-release in vitro, with aggregation mediated by possible joule heating in silver colloids. Exaggerated aggregation and SPR red shifting is expected for DTT-treated samples. Fluorescence data for SNP separated TYE563-labeled oligonucleotide ligands reveal effective purification (sample 2) and photo-release (sample 3) of ligands from SNP-oligo conjugates (Figure 2-9 b).

The maximum yields of photo-release at both low (302nm) and high (356nm) wavelength ranges reached 50-60% of total ligand, based upon comparison of photo-released TYE563-labeled oligonucleotide fluorescence intensity to that of subsequent DTT-released ligand, where DTT removes all remaining oligonucleotides following photo-exposure. Light dose-response plots reveal a traditional progression of photo-conversion over time for nitrophenylethyl-containing photolabile oligonucleotides (Figure 2-10). Experimentally calculated functional quantum yields of SNP-tethered NPE linkers (Table 2-1) indicate that at a photo-irradiation wavelength of 302nm, a particle-to-NPE-linker distance of 2-3nm displays a higher quantum yield than the greater distances of 5-6nm particle-to-NPE-linker spacing for the 10mer linker oligo. Future studies are recommended for optimization of surface photochemistry and ligand photo-release. A functional quantum (Φfa) yield was deemed appropriate for evaluation of conversion of an oligonucleotide synthesized with an internal NPE linker into two separate oligonucleotide segments, as this is a measure of efficiency of ligand release from SNP-oligo conjugates and involves processes not considered in standard photochemical compound quantum yields.
Figure 2-9 – Photo-release from SNP surfaces. a) Normalized absorbance spectra for non-functionalized silver nanoparticles (SNP), purified photo-sensitive SNP-oligo conjugates (Fluor SNP Purified), light-irradiated SNP-oligo conjugates (Fluor SNP Flash), and SNPs following DTT-mediated removal of surface oligonucleotides (Fluor SNP DTT). b) Graph fluorescence intensity for TYE-labeled oligonucleotide removed from nanoparticle surfaces via centrifugation (1 & 2), flashing (3), and DTT treatment (4). Inset: Gel electrophoresis run of samples 1,2,3 & 4.

Figure 2-10 - Percent conversion of SNP-bound to photo-released oligonucleotide states. a) Comparison of NPE(10n)-oligo and TYE-NPE(1n)-oligo photorelease from the SNP surface at a photo-irradiation wavelength of 320nm b) Comparison of TYE-NPE(1n)-oligo photorelease from the SNP surface at a photo-irradiation wavelengths of 320nm and 365nm.
Table 2-1 - Photocleavage properties of SNP-bound photolabile oligonucleotides

<table>
<thead>
<tr>
<th>Condition/Oligonucleotide</th>
<th>Extinction (ε) M⁻¹ cm⁻² @320nm</th>
<th>Extinction (ε) M⁻¹ cm⁻² @365nm</th>
<th>Quantum Yield (ϕ) @320nm</th>
<th>Quantum Yield (ϕ) @365nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized Fluor Oligo (1b)</td>
<td>3520</td>
<td>660</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>Immobilized PC Oligo (2a)</td>
<td>3520</td>
<td>660</td>
<td>0.09</td>
<td>---</td>
</tr>
</tbody>
</table>

2.4.7 Confocal Microscopy Analysis of SNP-conjugate Delivery and Photo-release Profile

Confocal microscopy results display effective intracellular delivery of SNP-oligonucleotide conjugates and photo-release of oligonucleotide ligands *in situ*. Delivery of SNP-oligo conjugates to the intracellular environment was achieved without the use of extraneous transfection vectors (Figure 2-11, SNP-oligo conjugate images 3a,b,c,d). This is opposed to naked DNA oligonucleotides, which traditionally require a transfection carrier such as a cationic lipid or polymer to facilitate intracellular delivery (Figure 2-11 TYE-oligo + TurboFect images 4a,b,c,d). Localization of SNP-oligo conjugates in the intracellular space was confirmed via 3-dimensional reconstruction of thin-slice confocal image stacks (*supplemental information* Figure 2-15- Figure 2-16). Overlay of particle reflectance at 488nm with TYE-labeled oligonucleotide fluorescence (549nm/563nm) indicates co-localization of oligonucleotide ligand with SNPs in the absence of UV-light exposure (Figure 2-11 3c). Naked SNPs were also observed to achieve high levels of cellular uptake, making the argument for transmembrane transportation based primarily on the properties (size, morphology) of silver nanoparticles alone. Naked and functionalized SNPs were found to reside primarily in the cytosolic environment of treated HeLa cells, as opposed to other areas not desired in mRNA-targeting antisense applications, such as nuclear compartments.
Figure 2-11 - SNP-Fluor oligo conjugate delivery to HeLa cells. Control (1) vs. naked SNP treated (2) vs. SNP-TYE-oligo conjugate treated (3) vs. TYE-oligo treated (4) HeLa cells. Images depict (a) silver particle fluorescence (488nm/520nm), (b) TYE fluorescence (549/563nm), (c) Reflectance + Fluorescence overlay, and (d) Brightfield views. SNPs exhibit a wide range of fluorescence emission, explaining appearance of faint silver signal in the TYE563 channel (2b).

Intracellular redistribution of labeled oligonucleotides upon photo-exposure of SNP-oligo conjugates was also demonstrated via confocal microscopy (Figure 2-12). In all non-photo-exposed samples, TYE-labeled oligonucleotide fluorescence co-localized with silver nanoparticle fluorescence (Figure 2-12 panel 1a,b,c), in a punctuate pattern within the cell (see schematic I in Figure 2-12). Particle fluorescence is demonstrated in green for ease of
visualization of overlay (yellow) regions. Co-localization of TYE563 and silver fluorescence indicates that conjugates are stable and remain intact after 16-24hrs within a cellular environment. However, upon UV-irradiation of cells treated with photo-sensitive SNP-oligo conjugates, a diffusion of oligonucleotide fluorescence (Figure 2-12 panels 2,3) reflects release of functional ligands from particle surfaces and implicates increased availability of these oligonucleotides for binding to mRNA targets within the cytosol. Sequestration of oligonucleotides within the nucleus, often observed for cells loaded with oligonucleotides via commercially available lipid or polymer transfection agents, is not observed for photo-activated SNP-oligo conjugates.

**Figure 2-12** – Photoreleased SNP-Fluor oligo conjugates. I. Schematic of intracellular particle-bound oligonucleotide ligand (a) vs. photo-released ligand (b). II. Non-released (1) vs. photo-released (2,3) *ex vivo* SNP-fluor conjugates samples. Images depict (a) Particle fluorescence (488/520nm), (b) TYE fluorescence (549/563nm), (c) Fluorescence overlay, and (d) Brightfield views.
### 2.4.8 Antisense Activity of SNP-oligo Conjugates

Confocal microscopy results display the successful delivery and caging effect of silver nanoparticle carriers for antisense DNA oligonucleotides. SNP-bound ISIS 2302 antisense oligonucleotides, which target ICAM-1(CD54) mRNA, were shown to decrease the expression level of INF-γ upregulated CD54 upon external photo-activation, i.e. photo-release from silver particle surfaces within the intracellular environment (Figure 2-13). Successful knockdown is evidenced by reduced membrane labeling of a primary mouse anti-human CD54 APC-conjugated antibody in photo-exposed, SNP-oligo conjugate treated HeLa cells. This reduced labeling is apparent when we compare non-flashed (2d) and flashed (3d, 4d) cases, where cell membrane staining with APC (yellow) is indicative of CD54 expression levels. Diffuse, SNP photo-released TYE-oligo can be seen as diffuse cytosolic fluorescence delineated on the cell periphery by faint antibody staining (3d, 4d). It is important to note that the appearance of broadly fluorescent nanoparticles in the APC emission images can be differentiated from membrane antibody staining due to differences in localization. Overlay of SNP and TYE emission appears magenta, whereas overlay of SNP, TYE, and APC emission appears white. Naked SNPs at similar loading concentrations were shown to have no effect on CD54 expression in both non-UV-exposed and UV-exposed cases (data not shown). Non-caged free solution TYE-oligo was used as a control, loaded at high concentrations via TurboFect polymer transfection agent. Limited APC staining is apparent in these control samples, providing evidence that our modified TYE-labeled ISIS 2302 antisense oligonucleotide is active against ICAM-1.
Figure 2-13 – Antisense activity of photo-activated SNP-TYE-oligo conjugates. CD54-expressing HeLa cells without antisense treatment (1) are compared to SNP-TYE oligo conjugate treated cells in both non-activated (2) and photo-activated (3, 4) cases. TurboFect (TF) transfected TYE-oligo treated HeLa cells (5) are used as a control case. Images depict (a) silver particle fluorescence (488/520nm), (b) TYE fluorescence (549/563nm), (c) APC-conjugated anti-human CD54 fluorescence (633/680), (d) fluorescence overlay, and (e) Brightfield views. SNPs exhibit a wide range of fluorescence emission, explaining appearance of faint silver signal in the TYE563 channel (2b).

2.5 Discussion

The synthesis and in vitro/intracellular characterization of UV-photo-activated silver nano-carriers of antisense therapeutics has been accomplished. Moderately sized silver
nanoparticles (60nm in average diameter) were shown to be easily functionalized with thiol-capped nitrophenylethyl (NPE) photocleavable linker-containing oligonucleotides. The photochemistry of NPE was found to be fully functionally at traditional UV wavelengths when tethered close to optically active SNP surfaces. The enhancement factor of absorption, fluorescence, scattering, or photochemistry of any chromophore or photochemical compound at or near a silver surface will depend on particle shape and size, as well as the distance of the photoactive molecule from the particle surface[36]. The size factor was considered in our selection of appropriate SNP sizes for maximum efficiency of ligand fluorescence detection and nitrophenylethyl (NPE) photochemical processes near silver surfaces. Moderately sized particles (~50nm in diameter) have been found previously to display the most enhanced intensity of local fields and surface enhanced fluorescence (SEF)[31]. The distance factor is of particular importance in relation to photochemical processes of molecules directly adsorbed or tethered a short distance from the metal surface (<1nm), where the effective transfer of energy from the excited molecule to the metal surface, i.e. quenching, competes with the accumulation of energy by the molecule, often enhanced due to interaction with the surface[41]. Thus an important consideration in our studies on photo-release of therapeutics from silver surfaces was tailoring the distance of the NPE photo-cleavable linker from the SNP surface in order to strike a compromise between field enhancement (i.e. enhanced photochemistry) and nonradiative damping[9] or quenching processes. A separation of ~2-3nm has been found appropriate for efficient release of SNP-tethered oligonucleotide therapeutics using UV-light exposure doses non-toxic to a treated endothelial cell line. Distance-dependent photoreactivity of SNP-tethered photocleavable linkers could be further elucidated by a complete investigation of various particle-to-NPE-linker distances to determine optimal oligo design for photorelease. Optimal
distances for surface-enhanced photochemistry will depend on compound reaction rates and nonradiative decay rates to the metal surface[121]; orientation of photoactive compounds to the surface[128], and properties of the particles themselves including size and morphology[32]. However, favorable distances will theoretically reside within a range proportionate to the substrate particle radius, due to significant decay of near-field enhancements beyond this range.

We have used to our advantage the characterized broad range of SNP optical properties, including fluorescence in a standard FITC channel, for detection and confirmation of SNP-antisense conjugate delivery and intracellular drug release. The SNP-based delivery platform was shown to not only provide nuclease resistance of transported antisense therapeutics, but also enhance the cellular uptake and intracellular diffusion of their nucleic acid ligands upon photo-release from particle surfaces. The light-triggered increase in cytosolic distribution indicates augmented availability of oligonucleotide therapeutics for hybridization to mRNA targets, revealed by SNP-conjugate photo-activated ISIS2302 antisense activity against Intracellular Adhesion Molecule-1 (ICAM-1), a membrane protein over-expressed in the presence of particular cytokines in HeLa cells ([129]). Delivery enhancements, it is important to note, were accomplished in the absence of additional transfection vectors. Efficiency of SNP-mediated delivery of antisense oligonucleotides to the intracellular environment was found to vie with that of a commercial standard polyethyleneimine-based transfection agent, considering factors including amount, rate, and ease of drug delivery. These results corroborate previous studies showing enhanced delivery of SNP-oligo conjugates into cellular environments based on dense surface charges, associated salts, and adsorbed proteins in serum medias, with internalization exaggerated upon increasing oligonucleotide surface loading density. Cell culture assays demonstrate, in addition to successful delivery and release of SNP-bound antisense therapeutics,
effective caging of antisense activity for oligonucleotides attached to SNP surfaces in the absence of external photo-activation cues, whereupon therapeutic activity was restored. These silver nano-composite caging systems permit photo-controlled activation of gene silencing. With the advancement of new delivery agents and controlled targeting/release methods a prized goal in nanomedicine and antisense gene therapy technology[16], a novel silver antisense nano-carrier for photo-activated gene silencing holds high impact as a promising alternative to existing drug delivery platforms. New light-sensitive intelligent drug delivery systems are also of high interest, and our synthesis of a silver nano-conjugate caging platform for antisense oligonucleotides is a step in the direction toward multicomponent nano-systems for smart drug delivery with improved mechanical properties and precisely controlled activity in vivo[68]. The technology promises high impact in the fields of nanomedicine and antisense gene therapy.

2.6 Supplemental Information

2.6.1 Surface Coverage Quantification: TYE-oligo Calibration

SNP-oligo conjugate surface coverage was quantified using fluorescence calibrations (Figure 2-14) of TYE-NPE(1n)-oligo at known concentrations. Oligonucleotide at given concentrations in 100ul volumes were analyzed for fluorescence intensity values (540/563nm). These values were then used to evaluate experimental quantities of DTT-removed oligonucleotides from functionalized SNP surfaces.

2.6.2 SNP-Conjugate Delivery: Confocal 3D Reconstruction

Confocal z-direction stacks were taken of SNP-TYE-oligo conjugate treated cells in order to verify intracellular localization. Select slices are shown (Figure 2-15) which confirm that particles are located in the cytosolic environment of imaged cells. 3D reconstruction rotating images are also provided which reveal the three-dimensional SNP-oligo conjugate delivery
throughout a treated HeLa cell (Figure 2-16). Images were taken using a Leica DM IRE2 inverted microscope with a galvo-Z stage. Images were compiled and rendered into 3-dimensional projections using Leica Lite software.

![TYE-Oligo Fluorescence Calibration](image)

**Figure 2-14** – TYE-oligo calibration curve for surface coverage quantification

![Brightfield and TYE Emission Images](image)

**Figure 2-15** - Confocal stacks of HeLa cells treated with SNP-TYE-oligo conjugates. Shown are stack (1-11) and all-layer projection (12) images. Images depict a) brightfield and b) TYE emission (549/563nm) views.
**Figure 2-16** - Confocal 3D images of a single HeLa cell treated with SNP-TYE-oligo conjugates. Shown are stack (1-9) images which depict a) SNP emission (480/520nm) and b) TYE emission (549/563nm) views.

### 2.7 Conclusion

In conclusion, we have synthesized and characterized antisense silver nano-conjugates for enhanced delivery of antisense therapeutics and photo-activated gene silencing. Our designed SNP-oligo conjugates display desirable properties as drug delivery agents both *in vitro* and *ex vivo*. We hope in future studies to further characterize oligonucleotide linker photochemistry and the effect of SNP surface-enhancement properties on this chemistry, as well as investigate multi-component SNP-conjugate systems with increased protection and photo-activated antisense activity against a variety of gene targets. We believe our studies have contributed significantly to the investigation of metal nano-composites as improved drug delivery vehicles and antisense therapeutics. The developed technology may be used in laboratory settings for gene expression analysis and sensing applications, as well as in clinical settings for antisense therapy and study of genetic diseases.
2.8 References


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2007, New York: Springer.


Chapter 3. Conclusions and Future Work

3.1 Conclusion

We have accomplished the synthesis and *in vitro/ex vivo* characterization of a novel antisense drug delivery platform for photo-activated gene therapy based on silver nanoparticle composites. Important goals of the thesis project included: surface functionalization of SNPs with photolabile antisense oligonucleotides and characterization of composite stability, surface coverage, ligand protection, and photo-activation efficiency; optimization of surface photochemistry; evaluation of delivery and subcellular localization of silver nano-composites *ex vivo*; and finally investigation of light-triggered antisense activity of SNP-conjugates against ICAM-1 in a fluorescence detection based gene silencing assay in HeLa cells. The results of these experimental objectives give weight to the exploration of silver nano-composites as potent platforms for photo-activated gene silencing with unique optical properties and potential for surface-enhanced photo-release capabilities. Densely packed SNP-antisense conjugates where shown to provide protection to attached antisense DNA oligonucleotides, inactivating or ‘caging’ the nucleic acid ligands until light-triggered release of the active therapeutics from silver surfaces. Silver nanoparticle drug delivery conjugates were found to be non-toxic at sufficiently high concentrations to provide effective antisense activity. Our demonstration of silver nanoparticles in drug delivery and gene therapy applications is groundbreaking due to a previous bias against use of silver nanomaterials in these fields of study. We hope to pursue our research on SNP-antisense composites as multicomponent delivery/caging platforms for nucleic acid therapeutics.
3.2 Future Work

We hope to complete our studies on the silver nanoparticle antisense composites we have described with several investigations ranging from in vitro to in vivo characterization. The following investigations are proposed:

3.2.1 Long-term Effects of Oligonucleotide Silver Nano-composites on Cell Health.

Experiments will include viability studies on naked SNP and SNP-conjugate treated HeLa cells out to 48hr treatment times. We expect limited toxicity based on preliminary cell health analysis based on morphology of SNP exposure cells. Data will include fluorescent staining indicative of cell death or membrane compromised states, analyzed via confocal microscopy or flow cytometry.

3.2.2 Comparison of Silver Surface Tethered NPE (Nitrophenylethyl) Linker Photochemistry to That of NPE Compounds in Solution.

Methods will include HPLC analysis of TYE-NPE(1n)-oligonucleotide photo-cleavage in solution phase. HPLC will be required to discriminate between a 30mer intact oligo and photo-cleaved 29mer + 1mer components.

3.2.3 A Complete Study of NPE-mediated Ligand Photorelease Efficiency at Variable Particle to-NPE Linker Distances and Irradiation Wavelengths.

These investigations will help to evaluate the possibility of SNP surface-enhanced photocleavage and oligonucleotide photo-release at traditional ultraviolet as well as red-shifted excitation wavelengths. This potential surface-enhancement of photochemistry will be silver surface distance-dependent, requiring a fine tuning of NPE linker location along an SNP-attached oligonucleotide backbone.
3.2.4 Investigation of Various Fluorescence-independent Reporter Genes for Antisense Activity Evaluation (eg Luciferase).

As has been demonstrated in our results on naked SNP vs. SNP-oligonucleotide conjugate characterization, silver nanoparticles has significant fluorescence in a wide range of excitation and emission wavelengths. This characteristic can be problematic for multi-channel fluorescence-based detection assays, and can lead to reduced detection limit due to a base-line signal from fluorescent particles. Thus we propose to investigate alternative reporter gene systems for antisense activity evaluation of SNP-oligo conjugates in future work. These reporter genes may be chemical or phosphorescent in nature (ex. Luciferase), allowing for greater flexibility and improved detection limits in assays with fluorescent silver nanocomposites.

3.2.5 In Vivo Studies of Uptake and Fate of Injected SNP-antisense Conjugates.

We hope to further characterize our system for in vivo applications in future studies. We propose investigations on uptake and fate of SNP-antisense conjugates following injection in in vivo tissues. These will be performed in a suitable animal model, and fluorescent silver nano-conjugates may serve as biological labels for tracking injected samples.
Appendix. Materials and Protocols

A.I. Cell Culture Protocol

HeLa Splitting and Seeding Procedure

A. Feeding
1) Aspirate media
2) Add 5ml of fresh DMEM-RS+3%Fetal Bovine Serum per T-25 flask (doubling time = 24hrs)
3) Feed once a week for T-25 flask

B. Splitting cells (volumes are per one T-25 of confluent HeLa’s)
1) Aspirate media from flask to waste
2) Rinse w/ 5 ml CMF-PBS (Ca+ & Mg+ free) and swivel in flask
3) Aspirate off PBS to waste
4) Add 5 ml of Trypsin solution
   a. Wait ~10 minutes for cells to come off of the dish at room temperature. Gently rock flask periodically and observe under microscope to make sure the cells are detaching
5) Add 5 ml of DMEM-RS+3%FBS to inactivate trypsin
6) Transfer suspension to a 50ml centrifuge tube.
7) Spin down the cells at 1800rpm for 5 min on the centrifuge (Eppendorf 5702)
8) Decant supernatant
9) Resuspend in 5ml of DMEM-RS+3%FBS
   a. For Passaging:
      i. Add 5ml fresh DMEM-RS+3%FBS to a new T-25 flask
      ii. Add 3-5 drops of the cell suspension in the new T-25 flask for flask passaging
   b. For Experiment:
      i. Dilute cell media (1ml into 9ml fresh media) and seed 500ul diluted cell solution into 8-well chambered wells (LabTek chambered Slides, NUNC)

A.II. Cell Transfection Protocol

A. Transfection Protocol
1) Add TrojanPorter to 100ul of SFM (serum-free media) and incubate for 5min at RT
2) Complex DNA & TrojanPorter for ~15min at RT
3) Add polyplexes directly to 500ul of cell media in 8-well chambers → transfect for 16-24 hrs

A.III. Confocal Imaging Protocol

A. Confocal Protocol
1) Start-up: (Turn on laser fan and Ar/He laser key, allow lasers 15min for warm-up before use)
2) Start PC and turn on scanner
3) Turn on additional lasers if needed
4) Adjust laser intensity via knob on front panel (to ~ 40%)
5) Turn on microscope mercury lamp
6) Start-up Leica Software
   i. Open Beam control window: choose emission windows and gain settings
   ii. In confocal software, use Z-scan button to change to Z-wide
iii. Automatically change to oil objective (40x default)
iv. Switch to **Visual** for viewing on scope; **Scan** for imaging
v. Adjust laser intensity: start w/ low intensity (~20% or less) and adjust as necessary
vi. Adjust PMT gains (appropriate range 500-800) for all emission channels

7) Shut Down Procedure
i. Turn off microscope lamp
ii. Turn off lasers (MUST be turned off 15min prior to turning off laser fan)
iii. After 15min, turn off laser fan, scanner, and PC

A.IV APC-Antibody Staining Protocol

A. Antibody Staining (@16hrs post treatment)
1) aspirate cell media
2) wash in 500ul CMF PBS + 2% BSA
3) resuspend in 200ul OptiMem + 2% BSA
4) add 10ul APC-Anti-Human CD54 antibody to each well (leave 1hr in dark)
5) wash in 500ul CMF-PBS
6) resuspend in 500ul OptiMem for confocal imaging

A.V Hybridization Assay Protocol

A. In Solution Hybridization Assay Protocol (*samples in duplicate*)

Non-released Sample:
1) Purify TYE-SNPs (500ul): 1 x 20min @7000RPM
2) Add MB (5ul 1/10dil) & Hyb (*conditions = 15min 60degC + 5min RT + 5min 4degC*)
3) Purify unbound MB from fluor-SNPs (1 x 20min @7000RPM)
4) Release via DTT ⇒ purify released oligo/duplex from SNPs (centrifuge & amicon) ⇒
measure fluorescence and run gel (*compare ssDNA vs. oligo-MB duplex*)

Released Sample:
1) Purify TYE-SNP (500ul)
2) Release via flashing (6min GS)
3) Add MB (5ul of 1/10dil) and Hyb (*conditions = 15min 60degC + 5min RT + 5min 4degC*)
3) Purify unbound MB/duplex from flashed fluor-SNPs (1 x 20min @7000RPM)
5) Release via DTT ⇒ purify released oligo/duplex from SNPs (centrifuge & amicon) ⇒
measure fluorescence and run gel (*compare ssDNA vs. oligo-MB duplex*)

A.VI. Nuclease Digestion Assay

A. Nuclease Assay for SNP-oligo conjugates
1) Purify SNP-oligo conjugates and resuspend in DNase I NEB buffer
2) Prepare TYE-oligo solution in DNase I buffer (3 samples)
3) Add DNase I to sample solutions – incubate at 37degC for digestion intervals
4) Add STOP buffer (EDTA) to inactivate DNase I
5) Purify digested SNP-oligo conjugates (1x)
6) DTT-remove oligo from SNP-oligo conjugates
7) Amicon filter TYE-oligo samples to remove salt and reduce volume for gel
A.VII. SNP Synthesis Protocol

A. SNP Synthesis
1) Thorough cleaning of all glassware with ethanol
2) Add .09g silver nitrate to 500ml DI water in large beaker
3) Add .3g sodium citrate (1%) to 30ml DI water in small beaker
4) Bring silver nitrate to boil while stirring (95 deg C)
5) Add 10ml of 1% citrate at a rate of 0.5ml/min (dropwise)
6) Color will change at 2ml of added citrate (pale yellow)
7) Color will change at 5ml of added citrate (dark green)
8) Let solution boil 10min after addition of 10ml citrate
9) Cool solution to RT and quickly transfer to fridge in air-tight, dark conditions

A.VIII. SNP Functionalization Protocol

A. Reagents
1) Thiol-Oligo: MW = 9349.2g/mol; Amount of Oligo: 155.8nmol = 1.46mg; Stock : 4ug/ul = 1460ug/365ul = 426.849315uM
2) TYE-NPE(1n)-Oligo: MW = 10184 g/mol; Amount of Oligo: 91.6nmol = .93mg; Stock : 4ug/ul = 930ug/232ul = 392.688172uM
3) PC-NPE(1n)-Oligo: MW = 9693.5g/mol; Amount of Oligo: 117.2nmol = 1.14 mg; Stock : 4ug/ul = 1140ug/285ul = 411.2280702uM

B. 4500:1 oligo to nanoparticles ratio; 1ml 72pM colloid = 7.2 x10^14 mol nanoparticles; 0.324uM Oligo per 1ml silver colloid

C. Protocol
1) Add Thiol-modified or cyclic-disulfide-modified oligonucleotides to citrate-stabilized silver nanoparticles (~ .324 nmole oligonucleotide per 1 ml of 72 pM colloid)
2) After 3hrs, add 10ul of 10% SDS, 25ul phosphate buffer (0.1 M; pH = 7.4) to bring the mixture to 0.0025 M phosphate
3) After 12hrs, begin salt aging process: in 4hr intervals add 10ul aliquots of Tris-NaCl buffer (2M in DI water, pH = 8 – 8.5) to a final concentration of 160-200mM Tris-NaCl (8-10 additions of 10ul aliquots) or until conjugate colloid is fading due to functionalization (to lighter golden color)
4) Rock resulting mixture gently (lowest speed) over course of 48hrs
5) Purification via centrifugation (3x 7000RPM)
6) Pull supernatant off SNP pellet and resuspend in dilute (100mM) Tris-NaCl Buffer + 10%SDS

A.IX. Materials and Reagents

SNP Synthesis Reagents:
1) Silver nitrate – lab bench (Ammar)
2) Sodium citrate – lab bench (Ammar)
3) Nanoparticle solutions – old fridge
4) All glassware/stirbars for synthesis – Ammar lab bench
5) Tris-NaCl salt buffer and other salt solutions – cabinet in microfab room

Transfection Reagents:
1) TurboFect (stock solution in main grad fridge – bottom door shelf)
2) OptiMem (grad fridge)
DNA/Oligo Stocks:
1) Grad/Ugrad Freezer (top shelf, in labeled IDT packages; in Paige’s Oligo Stocks)

ICAM-1 Assay Reagents:
1) INF-γ (3/2010 stock) – Freezer top shelf, Paige’s Stocks)
2) AbCam APC-labeled Antibody – Main grad fridge top shelf in original packaging

Flashing cuvette: Paige’s cabinet in microfab room (2nd shelf from bottom in black case)
Blue/Green Bandpass filters for GreenSpot – on cart with GS

Other Fluorophores:
1) LysoTracker Red – Grad freezer top shelf, with General Fluors
Vita

Paige Brown was born in Indianapolis, Indiana, to Donald and Kyle Brown. She was homeschooled in Indiana while actively competing in the sports of gymnastics and diving. She started her education LSU in the Fall of 2004, as an LSU student athlete, and completed her bachelor of science degree in biological and agricultural engineering in May 2008. She subsequently started her master’s studies in biological and agricultural engineering in Dr. Monroe’s lab. Her future plans include a career in both the biotechnology industry and academics.