1. Introduction

Gold nanoparticles (AuNPs) have become increasingly popular in diagnostic and therapeutic applications due to the biocompatibility, unique optical properties, small size, and ease of molecular functionalization of nanostructured gold.\cite{1} Such biomedical applications require the introduction of AuNPs to biological fluids and tissues, and frequently depend on the internalization of these nanoparticles by mammalian cells. Optical imaging of overexpressed cancer-associated mRNA in live, intact cells has been accomplished with AuNPs conjugated to fluorescent DNA that is complementary to the target mRNA sequence.\cite{2} These molecular probes are internalized by cells with high efficiency and do not require external transfection agents. Fundamental investigations into the cellular uptake of these DNA functionalized AuNPs (DNA-AuNPs) inform our understanding of their biological fate and assists in the ultimate design and implementation of such functional AuNP systems for the molecular characterization of diseased tissues.

The extent and efficiency of cellular uptake is dependent in part on the physicochemical characteristics of the nanoparticle system,\cite{3} including particle size,\cite{4} shape,\cite{4a,5} surface charge,\cite{6} and surface chemistry.\cite{7} Significant interplay between these factors is frequently observed, making identification of the effect of individual components complex.\cite{3c,5b,8} Typically cells internalize positively charged nanoparticles in higher amounts than neutral or negatively charged particles. However, DNA-AuNPs composed of highly dense, negatively charged DNA oligonucleotide strands have been shown to successfully enter cells in significant amounts such that intracellular mRNA imaging and delivery of therapeutic nucleic acids is achieved.\cite{2,7} No consensus has been reached on how particle size affects the cellular accumulation of functionalized AuNPs. For example, Cho et al. observed maximum cellular uptake for small AuNPs (<30 nm), while Chithrani and Chan found maximum uptake for larger AuNPs (50–70 nm).\cite{4a,5b} These discrepancies are likely due to a complex relationship between the size, surface charge, and ligand chemistry of the individual functionalized AuNP systems. Although the surface functionality and chemistry of DNA-AuNPs has been explored,\cite{7b,4a,5} a systematic investigation
into the influence of nucleic acid conformation, particle size, and surface charge on cellular uptake has not been reported.

To understand which physicochemical properties affect the cellular accumulation of DNA-AuNPs, we prepared AuNPs conjugated to hairpin DNA (hDNA) to form hAuNPs, single stranded DNA (ssDNA) to form ssAuNPs, and double stranded DNA (dsDNA) to form dsAuNPs (Scheme 1). Inductively coupled plasma mass spectrometry (ICP-MS), flow cytometry, and confocal microscopy demonstrate that CaSki cells, a human cervical cancer cell line, accumulate these particles in different quantities. The results indicate that the greatest predictor of cellular accumulation is DNA-AuNP size. We establish that cellular internalization can be modulated through adjustment of nanoparticle size, which will allow for the design of DNA-AuNP diagnostic and therapeutic systems with optimal transfection efficiency and enhanced intracellular imaging or DNA delivery properties.

2. Results and Discussion

2.1. Gold Nanoparticles Conjugated to Hairpin, Single Stranded, or Double Stranded DNA Enter Cells in Different Quantities

Two types of DNA ligands are conjugated to 15 nm citrate-stabilized AuNPs through a 5′-thiol group: (i) a ten thymine spacer ligand (T-10) to prevent crowding of the longer oligonucleotides and (ii) a cyanine dye (Cy5) labeled oligonucleotide sequence of five base pairs, and a loop sequence of 19 nucleotides. The ssDNA has a ten thymine spacer region, a stem formation sequence of five base pairs, and a loop sequence of 19 nucleotides. The ss- and dsDNA sequences have a ten thymine spacer region followed by an additional region of 30 nucleotides. The 19 base loop region of hDNA and 30 base region of ss- and dsDNA are randomized sequences of the four DNA nucleobases (A, C, G, and T) designed to have no known homology to human RNA so these studies would be focused purely on cellular uptake, rather than RNA sensing. GenScript Sequence Scramble was used to randomize the sequences and the National Center for Biotechnology Information (NCBI) Nucleotide Basic Local Alignment Search Tool (BLAST) was used to minimize sequence homology to the human mRNA transcript database. Hairpin DNA, ssDNA, and dsDNA are attached to citrate-stabilized AuNPs via gold-thiol bonds to form hAuNPs, ssAuNPs, and dsAuNPs, respectively. To form dsAuNPs, the complementary oligonucleotide (30 bases in length) is added with the thiolated strand in equimolar amounts during synthesis.

In the absence of cells, the inherent fluorescence of the DNA-AuNPs varies, likely as a result of the conformation of the DNA on the particle surface (Figure S1a, Supporting Information). The hAuNP is designed so that the fluorophore is held close to the gold surface by the stem-loop conformation of the DNA, resulting in the lowest observed fluorescence. The ssAuNP is formed from DNA of significant length, which can sample all energetically available random coil conformations. This could bring the 5′-fluorophore close to the gold surface, producing the observed quenching. The dsAuNP exhibits high fluorescence due to the double helical conformation that forces the fluorophore away from the gold surface, where the quenching efficiency is lower. Measurements of the hydrodynamic diameter reveal that hAuNPs and ssAuNPs have similar sizes of ≈31 nm (Figure S1b, Supporting Information). This small size is likely due to the folding of the DNA, whether forced by design (hAuNP) or sampled as the lowest energy conformation (ssAuNP). The dsAuNPs have a much larger hydrodynamic diameter of 66 nm due to the fully extended double helix. All three DNA-AuNPs have a similar zeta potential around −30 mV (Figure S1c, Supporting Information).

Confocal microscopy demonstrates that all of the DNA-AuNPs readily enter and localize to the cytoplasm of CaSki cells, a human cervical carcinoma cell line, without the need for transfection agents and within a few hours of introduction to the cell media (Figure 1). Z-stack images reveal that DNA-AuNP fluorescence (red) is observed throughout the volume of the cytoplasm, rather than solely localizing at the surface of the external cell membrane. These images also show that internalization of DNA-AuNPs occurs heterogeneously (Figure S2, Supporting Information), which is also supported by flow cytometry data, where the single cell population exhibits a distribution of fluorescence (see below). At the resolution and slice thickness of these images, we observe no DNA-AuNPs...
entering the nuclear compartment; however, the particles do tend to collect in the perinuclear region of the cytoplasm. DNA-AuNPs are known to nonspecifically enter a number of cell lines, although the exact mechanism is unknown. Previously our group utilized hAuNPs to fluorescently label viral mRNA in living cells infected with respiratory syncytial virus (RSV).\textsuperscript{[2c]} Measurement of RSV-specific hAuNP fluorescence in infected cells indicated that the nanoparticles are present in the cytoplasm, rather than contained within endocytic vesicles. Further exploration revealed that endocytic inhibitors do not significantly alter cellular uptake. Rather, modulators of cell membrane fluidity were found to have the most profound effect on internalization of hAuNPs, indicating that an unidentified nonendocytic process is facilitating uptake.

Cells incubated with hAuNPs, ssAuNPs, or dsAuNPs were evaluated for fluorescence by flow cytometry (Figure 2a). Cells treated with dsAuNPs have a histogram shifted to lower fluorescence values compared to cells treated with hAuNPs or ssAuNPs, suggesting lower amounts of transfection. The mean fluorescence for each cell population is given in Figure S3 (Supporting Information). Control cell viability studies were performed by the Trypan blue exclusion method (Figure S4, Supporting Information). The cells maintained ≥ 95% viability, which is consistent with previous results that showed no cytotoxicity of hAuNP-treated cells using fluorescent stains and flow cytometry.\textsuperscript{[2c,d]} ICP-MS provides aggregate data about the average cellular uptake of DNA-AuNPs by measuring the amount of gold internalized by the cells. To normalize across samples, the amount of internalized AuNPs was calculated on a per cell basis (see the Supporting Information for detailed calculations). ICP-MS analysis confirms that cells incubated with hAuNPs and ssAuNPs have significantly more (≈2x) AuNPs per cell compared to cells incubated with dsAuNPs (Figure 2b) (\( p < 0.05 \)). Differences in the cellular uptake of AuNPs functionalized with ssDNA and dsDNA have been observed previously, but to our knowledge, no explanation has been proposed.\textsuperscript{[12]} The levels of AuNPs per cell measured here (≈1.8 × 10\(^5\)) is comparable to previous reports that employed higher concentrations of nanoparticles and shorter incubation times.\textsuperscript{[7e]} Using low \textmu m concentrations, the transfection of DNA-AuNPs reaches the same order of magnitude as that of Narayan et al. by utilizing longer time scales, indicating that fine control over the accumulation of DNA-AuNPs in cells can be exerted by modifying the nanoparticle concentration and cell exposure time.

The internalization of nanoparticles over time was explored by incubating cells with hAuNPs, ssAuNPs, or dsAuNPs for 2–24 h. Cellular accumulation was measured by ICP-MS and flow cytometry (Figure 3a and Figure S5, Supporting Information). The cells internalize increasing quantities of DNA-AuNPs over time and a line of best fit was calculated using a linear regression between 2 and 10 h of incubation time. The slope of this line indicates that the DNA-AuNPs have different rates of uptake. hAuNPs and

![Figure 1. DNA-AuNPs localize within the cell. Confocal microscopy demonstrates that DNA-AuNPs are internalized into the cytosol of CaSki cells, rather than adsorbing to the cell membrane. Side and top views show the fluorescence in the z-direction, which reveals that DNA-AuNPs are found throughout the entire volume of the cell. Red: Cy5 labeled DNA-AuNPs. Green: SYTO13 nucleic acid stain.](image1)

![Figure 2. CaSki cells internalize DNA-AuNPs differently. a) Flow cytometry demonstrates that cells incubated with hAuNPs and ssAuNPs have populations shifted to higher fluorescence compared to cells incubated with dsAuNPs. These results suggest that cells internalize the three DNA-AuNPs differently. b) ICP-MS analysis of the gold concentration confirms that cells internalize dsAuNPs in significantly lower quantities than hAuNPs and ssAuNPs. Error bars represent standard deviation from three biological replicates. *\( p < 0.05 \).](image2)
ssAuNPs possess the fastest rate of uptake at 8000 ± 1000 nanoparticles per cell per hour, followed by dsAuNPs at 2830 ± 260 DNA-AuNPs per cell per hour. The rate at which nanoparticles enter the cell directly determines the final amount of DNA-AuNPs found inside; therefore, the reduced rate of uptake of dsAuNPs contributes to the lower intracellular levels of these nanoparticles. Flow cytometry shows that the mean fluorescence of the single cell population saturates between 10 and 24 h of incubation time, which may be due to self-quenching of the fluorescent DNA upon reaching a critical intracellular nanoparticle concentration. It is also possible that DNase degradation occurs within the cell causing a separation of Cy5 from the AuNP that results in recycling of fluorescent fragments out of the cell, while the AuNP core remains internalized, as proposed by Wu et al.\(^{[13]}\). The saturation of fluorescence observed here may be due to equilibrium between the processes of DNA-AuNP uptake and expulsion of fluorescent degradation products. By ICP-MS analysis, the levels of AuNP per cell do not saturate over 24 h, similar to previous reports that did not observe saturation in cells exposed to high concentrations of DNA-AuNPs over shorter time periods.\(^{[14]}\) It is possible that saturation of cellular uptake would be observed with longer exposure times. However, this is difficult to assess because the nanoparticles are transferred to daughter cells during cell division if exposure periods are longer than the cell doubling time are used.\(^{[15]}\) Additionally, at 24 h of incubation time, each cell has accumulated an average of almost two million hAuNPs or ssAuNPs, each of which has ≈80 functional DNA strands attached, which should be sufficient for imaging or therapeutic applications, negating the need to achieve saturating conditions.

To determine if intracellular DNA-AuNPs exit cells, particles were loaded into the cells over a 16 h incubation period. The cells were washed, fresh culture media was added, and the cells were incubated for an additional 2–8 h, which was followed by ICP-MS analysis. All three DNA-AuNPs have ≈100% retention in cells after removal of the extracellular AuNP solution and are not excreted in significant amounts when each time point was compared to the initial AuNP accumulation at 0 h (Figure 3b) (\(p > 0.05\)). These data correlate well with previous reports, which found that the quantities of functionalized AuNPs that are exocytosed are often less than 20% of the internalized nanoparticles.\(^{[13,16]}\) Wu et al. proposed that the high retention of functionalized AuNPs within cells was due to loss of stabilizing ligands, aggregation of the particles, and subsequent loss of biological recognition of the AuNPs, which prevents them from being excreted.\(^{[13]}\)

### 2.2. Addition of Complementary Oligonucleotide Strands to ssAuNPs and hAuNPs Reduces the Cellular Uptake of These Particles

To determine the impact of DNA conformation on cellular internalization, complementary strands were added to hAuNPs and ssAuNPs to form “complemented” versions of these particles. It is hypothesized that complemented DNA-AuNPs will behave like dsAuNPs when introduced to cells. ICP-MS shows that hAuNPs, ssAuNPs, and complemented hAuNPs are internalized at higher levels than complemented ssAuNPs and dsAuNPs (Figure 4a). Complemented ssAuNPs have similar cellular accumulation to dsAuNPs because these two nanoparticles exhibit indistinguishable physicochemical properties (Figure S6, Supporting Information). When analyzed by flow cytometry, cells incubated with hAuNPs and ssAuNPs attain higher fluorescence than cells incubated with complemented ssAuNPs and dsAuNPs, which correlates to the ICP-MS results (Figure 4b and Figure S7, Supporting Information). Cells treated with complemented hAuNPs have an intermediate fluorescence but high AuNP per cell content. This likely occurs because only a fraction of the hDNA on these particles has hybridized to the complementary DNA, as suggested by the low inherent fluorescence of complemented hAuNPs compared to dsAuNPs, as well as the high heterogeneity in hydrodynamic diameter (Figure S6a,c, Supporting Information).

Confocal microscopy provides an additional method to evaluate the cellular uptake of hAuNPs, ssAuNPs, dsAuNPs, and their complemented versions. After 4 h of incubation time, cells incubated with hAuNPs and ssAuNPs exhibit...
qualitatively brighter fluorescence than cells incubated with dsAuNPs, complemented hAuNPs, or complemented ssAuNPs (Figure 4c). Across the ICP-MS, flow cytometry, and confocal microscopy data, we generally observe agreement in the differences in DNA-AuNP cellular uptake measured by these three orthogonal techniques. Changing the DNA conformation on the particle surface affects the cellular uptake properties of these DNA-AuNPs. However, differences in cellular uptake cannot be definitively attributed to DNA conformation because particle size has not been controlled for in this set of DNA-AuNPs.

2.3. DNA-AuNP Size Determines the Extent of Cellular Accumulation

Particle size plays a large role in the cellular uptake properties of a variety of nanoparticle systems. To determine the influence of size, DNA-AuNPs are designed with (i) increasing lengths of DNA conjugated to AuNPs of uniform diameter or (ii) AuNP cores of increasing diameter conjugated to a single length of DNA.

2.3.1. Effect of Fluorescent DNA Length on the Cellular Uptake of DNA-AuNPs

These oligonucleotides are based on the design of the single stranded and double stranded DNA used previously. The original 40 base DNA strand was systematically shortened by eight bases to produce sequences of 16, 24, and 32 bases in length (see Table S2, Supporting Information, for sequence information). These ssAuNPs and dsAuNPs were synthesized analogously to previous DNA-AuNPs. An increase in the native fluorescence and hydrodynamic diameter of the dsAuNPs compared to their corresponding ssAuNPs of
identical sequence length confirms that dsDNA was formed on the particle surface (Figure S8a,b, Supporting Information). For the smaller DNA-AuNPs (ss16- and ss24-DNA), changes in fluorescence and hydrodynamic diameter are small, which we attribute to the inability of the short ssDNA to fold into a random coil, resulting in a strand that does not change in size with the addition of a complementary DNA strand. The extended length of the ss32 and ss40 strands of DNA allows for sampling of different conformations, which results in decreased fluorescence and size as the oligonucleotide folds. The larger size and higher fluorescence of ds32-AuNPs and ds40-AuNPs result from the adoption of a double helical conformation. All the DNA-AuNPs have statistically similar zeta potentials (≈−28 mV), regardless of DNA length or hybridization state (Figure S8c, Supporting Information). We hypothesize that this uniformity in zeta potential occurs because the DNA phosphate backbone becomes saturated with sodium ions during synthesis so as to achieve a similar surface charge across all types of DNA-AuNPs.

The extent of internalization of these eight DNA-AuNPs is dependent on the hydrodynamic diameter of the particle construct (Figure 5). Due to the folding of the DNA, the DNA-AuNPs separated into two size groups centered around 30 and 60 nm in diameter. The smaller DNA-AuNPs had higher levels of uptake than the larger DNA-AuNPs (p < 0.05). Zeta potential measurements did not correlate with cellular uptake, as was expected from the uniform zeta potentials of this group of DNA-AuNPs (Figure S9a, Supporting Information); therefore, surface charge does not influence the internalization of these DNA-AuNPs.

Oligonucleotide density of DNA-AuNPs has previously been shown to influence cellular uptake.[15] From our results, it is difficult to draw definitive conclusions about the role of DNA density in cellular uptake. However, we observe that (i) for DNA-AuNPs with significantly different DNA loading densities, there is no significant difference in cellular uptake (Figure S10a, Supporting Information) and (ii) for DNA-AuNPs that have the same density of DNA loading, significant differences in AuNP accumulation could be achieved, which we attribute to DNA-AuNP size since all other variables are held constant (Figure S10b, Supporting Information). Because DNA density cannot reliably predict nanoparticle accumulation in cells, it is likely not a major determinant of cellular uptake properties.

For nanoparticles of identical hydrodynamic diameter and zeta potential, Massich et al. observed that ssAuNPs exhibited ≈20 times higher accumulation in cells than dsAuNPs.[12] Here, it is observed that for DNA-AuNPs 30 nm in hydrodynamic diameter, ssAuNPs only exhibit 1.25 times higher cellular uptake than dsAuNPs, indicating that the conformation of DNA plays a minimal role in determining the amount of internalized particles.

2.3.2. Effect of AuNP Core Diameter on the Cellular Uptake of DNA-AuNPs

In order to further elucidate the roles of size and DNA conformation, the hydrodynamic diameter of the DNA-AuNPs was changed by varying the size of the AuNP core. Gold nanoparticles of increasing diameter were conjugated with a sequence of fluorescent DNA 24 nucleotides in length. This DNA length results in DNA-AuNPs large enough to cover the size range of interest. During synthesis, the citrate-stabilized AuNP solutions were adjusted to concentrations with the same surface area (S.A.) mL⁻¹ values, so that each reaction had the same amount of gold surface area available for binding thiolated DNA. The resulting DNA-AuNPs have a hydrodynamic diameter range of 23–62 nm, with a uniform size distribution (Figure S11, Supporting Information). The fluorescence of these particles in phosphate buffered saline (PBS) demonstrates that DNA is conjugated to the nanoparticle; however, comparisons cannot be made across the DNA-AuNPs due to variability in the quenching efficiency of differently sized cores. Zeta potential measurements of these DNA-AuNPs range from −25 to −35 mV. The surface charge is largely determined by the diameter of the AuNP core, as shown by zeta potential measurements of citrate-stabilized AuNPs with increasing diameters (Figure S11c, Supporting Information).

The cellular uptake of DNA-AuNPs made with increasing sizes of gold cores is highly size dependent (Figure 6). DNA-AuNPs with 10 nm cores result in the highest cellular uptake, and internalization decreases as the hydrodynamic diameter of the DNA-AuNP construct increases. The level of AuNPs per cell plateaus at hydrodynamic diameters larger than 50 nm where the difference in AuNP internalization is no longer significant (p > 0.05). For these nanoparticles, DNA density is also shown to play a minimal role in cellular uptake (Figure S10c,d, Supporting Information). Because surface charge trends with AuNP core diameter, the effects of size and surface charge cannot be completely disentangled, and surface charge may be involved in cellular uptake. However, it is worth noting that the DNA-AuNPs used in previous experiments have the same zeta potential (Figures S1c and S8c, Supporting Information), but exhibit different cellular uptake behavior, suggesting that the role of surface charge in cellular accumulation is probably minimal.

Although DNA-AuNPs with 40 and 50 nm cores have lower internalization compared to smaller nanoparticles,
the amount of AuNPs per cell is not insignificant and can be controlled by optimizing concentration and incubation time. At transfections of ~100 000 large DNA-AuNPs per cell and 350-750 fluorescent DNA per nanoparticle, there are 35-75 million functional DNA inside each cell. Therefore, even for applications that require nanoparticle constructs ≥50 nm in hydrodynamic diameter, cellular imaging or drug delivery should be feasible.

These results correlate with independent studies that show that cellular uptake of functionalized AuNPs varies inversely with particle size. Cho et al. reported that 15 nm gold nanospheres and 33 nm gold nanocages exhibited higher cellular internalization than 45 nm nanospheres and 55 nm nanocages, regardless of the functional group on the gold surface.\[4b\] Elbakry et al. demonstrated that the amount of AuNPs per cell decreased dramatically as the hydrodynamic diameter of coated AuNPs increased from 32 to 85 nm.\[4b\] These data, along with the results shown here, conflict with some reports, which state that for particles less than 100 nm, AuNPs around 50 nm in size are internalized by cells in higher amounts than smaller AuNPs.\[4a,4b\] It is difficult to explain these differences in reports of optimal size, but these varying results may be due to the use of different ligands or preparation methods.

4. Experimental Section

Cell Culture: CaSki cells (ATCC) were grown at 37 °C in a humidified atmosphere with 5% CO₂. The cell culture medium was RPMI 1640 (with L-glutamine) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units mL⁻¹), streptomycin (100 µg mL⁻¹), d-glucose (2.25 g L⁻¹), sodium pyruvate (1 × 10⁻³ M), and 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) (10 × 10⁻³ M). Cells were subcultured upon reaching ~80% confluence and media was replaced as needed.

Preparation of DNA Oligonucleotides: Lyophilized thiolated DNA sequences (Integrated DNA Technologies) were reconstituted in diethanol (0.1 M; DTT) in dibasic phosphate solution (0.1 M; pH 8.3) to reduce disulfide bonds. Following a one-hour incubation, the DNA was desalted and washed twice with Tris-Ethylene-diamine tetraacetic acid (TE) buffer using the manufacturer's protocol for Amicon Ultra 3K molecular weight cutoff filters (EMD Millipore). The DNA was resuspended in TE buffer and the concentration was determined by UV absorbance at 260 nm using a BioTek Take3 Micro-Volume Plate and a BioTek Synergy H4 Hybrid microplate reader. All nonthiolated DNA sequences were resuspended in TE buffer and the concentration was determined by UV absorbance. All sequences were adjusted to an appropriate working concentration using TE Buffer and stored at ~80 °C until use.

DNA-AuNP Synthesis: 10, 15, 20, 40, and 50 nm AuNPs (Ted Pella) were adjusted to the same S.A. mL⁻¹, 4.566 × 10⁻⁶ nm² mL⁻¹, using molecular biology grade water. Aliquots of 1 mL were supplemented with 0.1% (v/v) Tween 20. The thiolated ten thymine spacer ligand (T-10) was conjugated overnight at a final concentration of 0.1 × 10⁻⁶ M. Fluorescent DNA was added at

3. Conclusion

Careful isolation and elimination of the effects of nanoparticle characteristics on cellular internalization allow us to determine that the overall size of a DNA-AuNP construct is the primary characteristic that dictates the cellular uptake properties of this nanoparticle system. ICP-MS analysis of cells incubated with varying sizes of DNA-AuNPs supports this conclusion, as the average amount of AuNPs internalized per cell is highly dependent on the hydrodynamic diameter of the functionalized nanoparticle. The size of the DNA-AuNPs also determines the rate of cellular uptake with small nanoparticles exhibiting a rate of uptake almost three times that of larger nanoparticles. Confocal microscopy shows that DNA-AuNPs are truly internalized, rather than adhering only to the external cell membrane. Cells do not distinguish DNA-AuNPs by the conformation of DNA, as evidenced by data showing that ssAuNPs and dsAuNPs of similar size are internalized in similar quantities. Although it is difficult to completely disentangle the effects of nanoparticle size, DNA density, and surface charge, cells are shown to accumulate nanoparticles with the same surface charge or DNA density in significantly different amounts. This indicates that another factor is driving internalization, supporting our hypothesis that compared to nanoparticle size, surface charge and DNA density play a minor role in determining cellular uptake properties. These results contribute to our understanding of how DNA-AuNPs interact with the cell and what properties of the nanoparticle system are most important in the cellular internalization process. We demonstrate that we can exert control over the rate of uptake and the final accumulation of functionalized AuNPs in cells by adjusting the nanoparticle size, particle concentration, and cell exposure time, although it may be difficult to generalize these results to other ligand types or nanoparticle systems. Due to the ease of modulating nanoparticle size, these studies will inform the rational design of intracellular AuNP probes that possess optimal cellular transfection efficiencies, enhancing the ability to perform imaging or therapeutic applications on faster time scales with lower concentrations of DNA-AuNPs.
a final concentration of 0.3 × 10⁻⁶ μM and conjugated overnight. For dsAuNPs, the thiolated and complementary strands were added to the nanoparticles sequentially and allowed to conjugate and hybridize overnight. The solutions were buffered to pH 7.0 with phosphate buffer (10 × 10⁻³ M), brought to a final concentration of 0.1 M NaCl, and allowed to incubate for 24 h. The solutions were adjusted to 0.2 M NaCl and incubated for 24 h. A final NaCl addition resulted in a concentration of 0.3 M NaCl and the solutions were incubated overnight. All conjugations and incubations were performed with rotation in the dark. The resulting DNA-AuNPs were purified and concentrated through three rounds of centrifugation and resuspension in PBS. Centrifugation was performed at 21 100g for 20 min for 15 and 20 nm AuNPs; 16 100g for 45 min for 10 nm AuNPs; and 5000g for 30 min for 40 and 50 nm AuNPs. To prepare complemented DNA-AuNPs from ssAuNPs and hAuNPs, DNA-AuNPs were diluted to 100 fl DNA strands per particle. Reported values represent the mean ± SD for three biological replicates with analytical triplicates. For experiments measuring cellular uptake over time, reported values represent mean ± SD for two biological replicates with analytical triplicates, and the sample size is sufficient to provide the necessary statistical rigor.

**ICP-MS Analysis of Cellular Uptake:** CaSki cells were seeded in 12 well plates and allowed to adhere. For all experiments, cells were treated with 1 × 10⁻⁹ M (6 × 10¹¹ nanoparticles mL⁻¹) DNA-AuNPs prepared in fresh cell media. To measure the cellular uptake over time, cells were treated for 2, 4, 6, 8, 10, and 24 h. For all other uptake experiments, the incubation time was 24 h. The AuNP solution was removed and the cells were washed three times with PBS. The cells were detached using trypsin, collected in 15 mL conical tubes, stained with Trypan blue to assess cell viability, and counted using a Countess II automated cell counter (Life Technologies). Centrifuging at 300 g for 10 min pelletted the cells and the remaining media was completely removed. The cell pellet was digested overnight in 0.8 mL of aqua regia (Warning: extremely corrosive, handle with caution) followed by dilution to 6 mL with deionized water (18.2 MΩ cm). A Thermo Element 2 high resolution inductively coupled plasma mass spectrometer (ICP-MS, Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ionization (ESI) auto sampler (Elemental Scientific, Omaha, NE) was used to quantify the gold in the samples. Samples were taken by self-vaspiration via a 0.50 mm ID sample probe and capillary to the spray chamber. Gold was quantified by measuring the ¹⁹⁷Au isotope at medium resolution (R = 4300). The amount of AuNPs per cell was calculated as described in the Supporting Information. Typically, reported values represent mean ± SD for three biological replicates with analytical triplicates. For experiments measuring cellular uptake over time, reported values represent mean ± SD for two biological replicates with analytical triplicates, and the sample size is sufficient to provide the necessary statistical rigor.

**Flow Cytometry:** CaSki cells were seeded in 12 well plates, allowed to adhere, and treated with 1 × 10⁻⁹ M (6 × 10¹¹ nanoparticles mL⁻¹) DNA-AuNPs prepared in fresh cell media. Unless otherwise noted, cells were treated with DNA-AuNPs for 24 h. To measure the cellular uptake over time, cells were treated for 2, 4, 6, 8, 10, and 24 h. Following treatment, the AuNP solution was removed and the cells were washed three times with PBS. The cells were detached using trypsin and 20 000 events were collected on a BD LSRII flow cytometer with excitation at 633 nm. Unstained cells that were not exposed to DNA-AuNPs were analyzed as a negative control. To study the uptake over time, cells were detached with trypsin, fixed in 2% paraformaldehyde for 15 min at 4 °C, washed by centrifugation at 300g for 5 min, and resuspended in PBS before analysis. For all other experiments, cells were not fixed for analysis. Typically, reported values represent mean ± SD for three biological replicates with analytical triplicates. For experiments measuring cellular uptake over time, reported values represent mean ± SD for two biological replicates with analytical triplicates, and the sample size is sufficient to provide the necessary statistical rigor.

**ICP-MS Analysis of Cellular Excretion:** CaSki cells were seeded in 12 well plates and treated for 16 h with 1 × 10⁻⁹ M (6 × 10¹¹ nanoparticles mL⁻¹) DNA-AuNPs prepared in fresh cell media. The media containing excess AuNPs was removed and the cells were washed three times with PBS and incubated in fresh media containing no DNA-AuNPs for 2, 4, 6, and 8 h. See the Supporting Information for the AuNP per cell calculations. The percent AuNP retention was calculated by dividing the AuNPs per cell at a given time point by the AuNPs per cell at 0 h postmedia replacement. Reported values represent mean ± SD for three biological replicates with analytical triplicates.

**Confocal Microscopy:** CaSki cells were seeded in the wells of an eight well μ-slide (ibidi USA, Inc). DNA-AuNPs were added at 1 × 10⁻⁹ M (6 × 10¹¹ nanoparticles mL⁻¹) DNA-AuNPs prepared in fresh cell media. The media containing excess AuNPs was removed and the cells were washed three times with PBS and incubated in fresh media containing no DNA-AuNPs for 2, 4, 6, and 8 h. See the Supporting Information for the AuNP per cell calculations. The percent AuNP retention was calculated by dividing the AuNPs per cell at a given time point by the AuNPs per cell at 0 h postmedia replacement. Reported values represent mean ± SD for three biological replicates with analytical triplicates.

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