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Oligonucleotide Loading Determines Cellular Uptake of DNA-Modified Gold Nanoparticles

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ABSTRACT

The cellular internalization of oligonucleotide-modified nanoparticles is investigated. Uptake is dependent on the density of the oligonucleotide loading on the surface of the particles, where higher densities lead to greater uptake. Densely functionalized nanoparticles adsorb a large number of proteins on the nanoparticle surface. Nanoparticle uptake is greatest where a large number of proteins are associated with the particle.

The introduction and development of the oligonucleotide−nanoparticle conjugate has led to applications in molecular diagnostics,[1,2] materials synthesis,[3,4] and gene regulation.[5] In the case of therapeutic applications, the use of oligonucleotides for genetic regulation holds promise for both the investigation of gene function and the treatment of disease,[6] and thus developing new agents to both effectively deliver and utilize oligonucleotides in cellular systems is important. Several key factors must be addressed to create highly potent therapeutic systems, including efficacy of the agents, mechanism of delivery across cell membranes, and toxicity of both the agents and transfection systems.[7,8]

We have recently reported the ability of oligonucleotide-functionalized gold nanoparticles to act as both a cellular transfection and genetic regulation entity.[5] Specifically, antisense oligonucleotide-modified gold nanoparticle agents (ASNPs) retain their oligonucleotide shell under cell culture conditions, bind their complements with high binding constants,[9] are stable in physiological environments, resist nuclease degradation, enter a variety of cell types without the use of auxiliary reagents, and are easily enhanced through chemical modification to increase efficacy.[10] These so-called "antisense particles" overcome many of the challenges related to oligonucleotide transfection by taking advantage of cooperative properties conferred by immobilizing oligonucleotides into a dense monolayer on a gold nanoparticle surface.

A hallmark of these densely functionalized oligonucleotide-modified particles is their ability to enter a wide variety of cell types, which has qualitatively been demonstrated using confocal microscopy.[5] This unusual property is surprising and somewhat perplexing because ASNPs contain a densely packed monolayer of polyanionic DNA (19 pmol/cm^2) on the surface of each 13 nm gold particle (Au NP).[11] Generally, negatively charged species such as DNA require positively charged transfection agents for cellular internalization.[12−14] For example, the most common delivery systems employ cationic lipids and polymers that utilize positive charges to interact with cellular membranes.[15,16] Thus, no literature precedent exists to explain the cellular internalization of negatively charged ASNPs. Therefore, understanding the factors that control uptake of ASNPs may lead to new methods of transfecting cells with membrane-impermeable cargos including genes, small molecules, and drugs and inform future studies utilizing these agents. Herein, we quantify the uptake of ASNPs in a variety of cell models, characterize the interactions of these agents with proteins in the extracellular environment, and describe how their cellular internalization is determined by their oligonucleotide-modified surfaces and the ability of those surfaces to support protein adsorption.

To probe the generality of the uptake of ASNPs in cells, we examined a mouse cell line (C-166) and two human cell models (HeLa and A594). These cell lines were chosen to represent different species and inherent differences between cell and tissue types. In these experiments, particles were added directly to cell cultures in concentrations ranging from 1 to 60 nM, and the number of gold atoms in each sample was quantified 48 h after addition. Briefly, the cells were washed, collected by treatment with trypsin, counted, and digested in nitric acid, and the Au content of the cell

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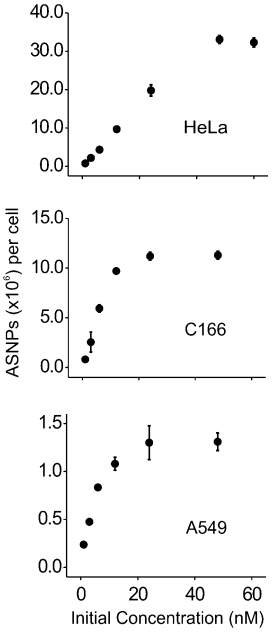


Figure 1. Cellular uptake of ASNPs. Analysis of ASNPs in three different cell types (HeLa, C166, A549) shows that the number of ASNPs per cell is dependent on initial particle concentration and cell type.

digest was determined by inductively coupled plasma mass spectrometry. The values obtained were used to calculate the number of ASNPs per cell (see Supporting Information). The results of these experiments demonstrate that while the uptake of ASNPs is universal across multiple cell types, the absolute number of nanoparticles varies by cell type by as much as a factor of 20 (Figure 1).

The amount of ASNPs found in each cell is proportional to the concentration of particles added to the media over the range of concentrations tested. Specifically, at low concentrations, uptake varies by concentration in a linear manner until a saturation point is reached at the highest concentrations tested (Figure 1). Additionally, the total number of ASNPs determined to enter or associate with each cell type is remarkably high, reaching up to 3×10^7 nanoparticles per cell in HeLa cells. Similarly, the maximum number of ASNPs is 1×10^7 and 1×10^6 nanoparticles per cell in C166 and A549 cells, respectively. For comparison purposes, others have observed from one thousand to five thousand nanoparticles per cell in an analogous cell-line for Au NPs that are not functionalized with DNA.[17] These experiments used a similar collection, washing, and mass-based quantification process and underscore the important contribution of the oligonucleotide-modified surfaces.

Because the number of ASNPs associated with each cell was found to be higher than reported values for other modified Au NP systems, we hypothesized that the oligonucleotides were strongly contributing to cellular uptake. To probe this contribution, we prepared particles with varying numbers of oligonucleotides by co-functionalizing them with OEG thiol diluent (Figure 2A). OEG was chosen because of its charge neutrality, water solubility, and its ability to passivate surfaces in a manner that resists adsorption of biological molecules.[18] It should be noted that diluent molecules must be included to keep the Au NP surface fully passivated and the particles stable. This OEG diluent allowed ASNPs to be synthesized with $0-80 \pm 2$ oligonucleotides per particle that were stable under all the conditions required for cellular culture. The stoichiometry during the functionalization process was varied to produce a range of DNA loadings (Figure 2A).

Quantification of the cellular uptake of these mixed monolayer particles demonstrated that uptake is highly dependent on the number of oligonucleotides immobilized on each ASNP. For example, with the A549 cell line (Figure 2B), which is qualitatively representative of the behavior of the C166 and HeLa cells (see Supporting Information, Figure 1), particles with lower oligonucleotide loadings are not readily internalized by cells. However, at loadings of 60 strands per particle cellular association reaches a maximum of approximately 1.3×10^6 ASNPs/cell and does not appreciably increase at higher oligonucleotide density. For comparison purposes, OEG-functionalized particles without oligonucleotides were also prepared and investigated under identical experimental conditions. While still exhibiting uptake (thousands of nanoparticles per cell), even at the highest (36 nM) concentrations examined, the uptake of fully OEG-functionalized particles were significantly lower than the ASNP particles (10^3 lower uptake) (Figure 2C).

In addition to quantitative measurements, we also investigated the size and surface potential of the particles before and after exposure to cell culture conditions to gain insight into how they were interacting with the extracellular environment. Dynamic light-scattering (DLS) measurements were performed to estimate the initial measured hydrodynamic

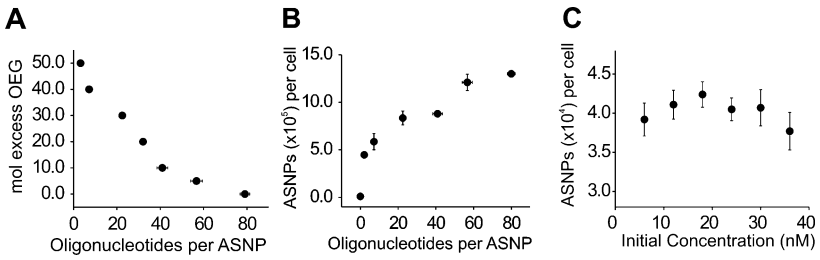


Figure 2. (A) Characterization of oligonucleotide loading on OEG:DNA particles. (B) Uptake as a function of the number of oligonucleotides per ASNP in A549 cells. The number of ASNPs per cell increases as the number of oligonucleotides increases (particle concentration in the media was 10 nM). (C) Au NPs functionalized with OEG show comparatively little uptake (A549 cells) even at high concentration additions.

Table 1. ASNP Characteristics before and after Media Exposure

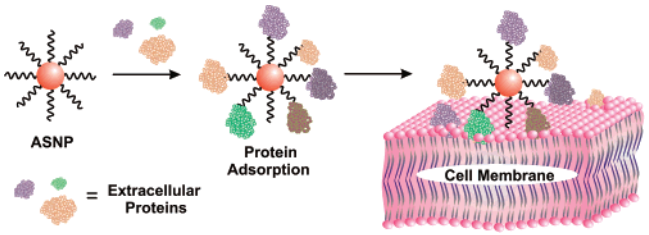
oligonucleotide strands/ AuNP	diameter (nm) before media	diameter (nm) after media	surface potential (mV) before media	surface potential (mV) after media	adsorbed proteins/ ASNP
79 ± 2	42 ± 1	76 ± 3	−21 ± 4	−13 ± 1	23 ± 3
32 ± 1	56 ± 2	77 ± 2	−36 ± 2	−24 ± 2	14 ± 1
7 ± 1	38 ± 1	50 ± 2	−34 ± 2	−27 ± 1	10 ± 1
0	27 ± 1	30 ± 1	−20 ± 1	−19 ± 1	2 ± 3

radius of the ASNPs. DLS data shows that the average diameter of an ASNP functionalized with only DNA (approximately 80 strands; 13 nm Au NP) is 42 ± 1 nm while that of a fully OEG-functionalized particle is 27 ± 1 nm. Interestingly, the average size of the DNA-functionalized particles increases to 76 ± 3 nm upon exposure to cell culture media, while the size of the OEG-functionalized particle remains relatively constant (Table 1). This observation suggests that in cell culture media alone, some components are attracted to the ASNPs, which results in an increase in size.

Zeta potential measurements indicate a change in surface potential that accompanies the size change of the ASNP, which we hypothesize is due to positively charged serum proteins binding to the DNA shell on the Au NPs (Table 1). Initially, the surface potential of the ASNPs in nonserum-containing media was −21 ± 4 mV. After exposure to media-containing proteins, the ASNPs became more positively charged (−13 ± 1 mV). In contrast, Au NPs functionalized with OEG did not show a change in surface potential after exposure to serum-containing media. While ASNPs appear to become associated with serum proteins, the OEG particles do not. This is consistent with the well-characterized passivation properties of OEG monolayers with respect to protein adsorption.[18] Note that the particles with OEG diluent molecules in Table 1 (32 strands or fewer) tend to have larger diameters than the ones with only DNA on the surface. The OEG likely lifts the DNA off the particle surface, thereby increasing the particle diameter and increasing the DNA contribution to the measured surface potential.

Further analysis using a fluorescence-based assay for protein quantification was carried out and confirmed that the observed size and surface potential changes were due to protein adsorption on the ASNP surface. Additionally, this assay allowed for an estimate of the number of proteins that are attached to each particle. In the case of a fully DNA-functionalized Au NP (80 strands per 13 nm particle), approximately 23 proteins remain attached to each particle after separation from the media (assuming an average protein is 60 kD). As the number of oligonucleotides per particle decreases, so does the number of proteins per particle (Table 1). These numbers may be interpreted as minimum values, as the washing process to remove unbound proteins could remove weakly bound proteins from the ASNPs. Nonetheless, the assay allows for comparison of particles functionalized with varying numbers of oligonucleotides, and it confirms that the density of oligonucleotides directly correlates with the number of proteins, and provides a possible reason for the increasing uptake of the ASNPs as a function of DNA loading. While others have looked at the contribution of nonspecific serum proteins to the uptake of citrate stabilized Au NPs,[19] the number of proteins as a determining factor in the uptake of Au NPs has not yet been established. Our data indicates that specific surface modification by oligonucleotides can be used to control the number of proteins and hence control cellular interactions of Au NP agents and perhaps materials in general. The measurements demonstrate that the quantity of ASNPs associated with each cell is significant and orders of magnitude larger than what has been observed for nonfunctionalized protein- or peptide-modified Au NPs.[20]

Scheme 1. Proposed Scheme for ASNP Cellular Uptake[a]



[a] Upon addition to the media, ASNPs bind extracellular proteins and allow the ASNP to interface with the cellular membrane. The number of proteins per particle correlates with particle uptake.

On the basis of literature precedent and the negative charge presented by the DNA functionalization, this uptake ability would not be anticipated. To test the contribution of the oligonucleotides present on the nanoparticle surface to their cellular uptake, the density of oligonucleotides on the surface was varied using OEG as a diluent. We found that even at high concentrations, Au NPs functionalized with only OEG showed comparatively little internalization by the cell models studied. Thus, in the co-functionalized particles the oligonucleotides provide the contribution to cellular internalization. By increasing the number of oligonucleotides, we observed increased uptake of the ASNPs with a maximum uptake reached at loadings of approximately 60 oligonucleotides per Au NP. These data show that the surface density of oligonucleotides mediates the amount of nanoparticles internalized by cells.

We also characterized the physical properties of the nanoparticle agents before and after exposure to the extracellular environment. Biophysical characterization revealed changes in charge and size of ASNPs after exposure to serum-containing media. However, in the case of OEG-coated particles, size and charge remained constant. Because cells will readily recognize certain proteins,[21] we hypothesized that the interaction of ASNPs with proteins is a possible mechanism of recognition and their subsequent internalization (Scheme 1).

Our data indicates that the number of proteins increases with the number of oligonucleotides on the surface, reaching a maximum of 23 proteins/particle. Further, their subsequent uptake correlates well with the number of absorbed proteins. The uptake plateau at 60 oligonucleotides per particle is

perhaps due to a saturation of proteins on the surface of the oligonucleotide layer. Beyond this point, additional oligonucleotides may confer no additional ability to recruit proteins. Compared to other particles and traditional transfection agents, the differences in uptake of ASNPs may be due to both the number and nature of the proteins that are attracted to the oligonucleotides on the ASNPs.

In summary, ASNPs have an extraordinary ability to enter cells. Their internalization is the result of several factors, including oligonucleotide density, concentration of ASNPs added, and the cell type. While further investigation will examine the mechanism of uptake of these materials, physical characterization of the ASNPs after exposure to cell culture conditions shows this uptake involves protein adsorption. The identity of the key protein(s) has not yet been determined but stands as an important challenge for the research community. Indeed, the discovery of the key protein(s) could lead to a universal coating to facilitate nanoparticle[22−28] and materials transfection in general. This work provides initial fundamental insight into the cellular internalization of oligonucleotide-modified nanoparticles and further offers design considerations for those wishing to exploit the properties of antisense nanoparticles, as well as other agents, for genetic regulation, intracellular detection, and therapeutics.

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Supporting Information Available: Materials and experimental procedures. Uptake data for OEG-ASNPs in C166 and HeLa cells. This material is available free via the Internet at http://pubs.acs.org.

References

(1) Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301* (5641), 1884−1886.
(2) Elghanian, R.; Storhoff, J. J.; Mucic, R. C.; Letsinger, R. L.; Mirkin, C. A. *Science* **1997**, *277* (5329), 1078−1081.
(3) Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. *Nature* **1996**, *382* (6592), 607−609.
(4) Alivisatos, A. P.; Johnsson, K. P.; Peng, X. G.; Wilson, T. E.; Loweth, C. J.; Bruchez, M. P.; Schultz, P. G. *Nature* **1996**, *382* (6592), 609−611.
(5) Rosi, N. L.; Giljohann, D. A.; Thaxton, C. S.; Lytton-Jean, A. K. R.; Han, M. S.; Mirkin, C. A. *Science* **2006**, *312* (5776), 1027−1030.
(6) Wang, L. X.; Prakash, R. K.; Stein, C. A.; Koehn, R. K.; Ruffner, D. E. *Antisense Nucleic Acid Drug Dev.* **2003**, *13* (3), 169−189.
(7) Stein, C. A.; Cheng, Y. C. *Science* **1993**, *261* (5124), 1004−1012.
(8) Roth, C. M. *Biophys. J.* **2005**, *89* (4), 2286−2295.
(9) Lytton-Jean, A. K. R.; Mirkin, C. A. *J. Am. Chem. Soc.* **2005**, *127* (37), 12754−12755.
(10) Seferos, D. S.; Giljohann, D. A.; Rosi, N. L.; Mirkin, C. A. *ChemBioChem* **2007**, *8* (11), 1230−1232.
(11) Hurst, S. J.; Lytton-Jean, A. K. R.; Mirkin, C. A. *Anal. Chem.* **2006**, *78* (24), 8313−8318.
(12) Boussif, O.; Lezoualch, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (16), 7297−7301.
(13) Bielinska, A. U.; Chen, C. L.; Johnson, J.; Baker, J. R. *Bioconjugate Chem.* **1999**, *10* (5), 843−850.
(14) Fraley, A. W.; Pons, B.; Dalkara, D.; Nullans, G.; Behr, J. P.; Zuber, G. *J. Am. Chem. Soc.* **2006**, *128* (33), 10763−10771.
(15) Remy, J. S.; Abdallah, B.; Zanta, M. A.; Boussif, O.; Behr, J. P.; Demeneix, B. *Adv. Drug Delivery Rev.* **1998**, *30* (1−3), 85−95.
(16) Lee, K. D.; Nir, S.; Papahadjopoulos, D. *Biochemistry* **1993**, *32* (3), 889−899.
(17) Chithrani, B. D.; Chan, W. C. W. *Nano Lett.* **2007**, *7* (6), 1542−1550.
(18) Prime, K. L.; Whitesides, G. M. *Science* **1991**, *252* (5009), 1164−1167.
(19) Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. W. *Nano Lett.* **2006**, *6* (4), 662−668.
(20) Liu, Y. L.; Shipton, M. K.; Ryan, J.; Kaufman, E. D.; Franzen, S.; Feldheim, D. L. *Anal. Chem.* **2007**, *79* (6), 2221−2229.
(21) Ryser, H. J. P. *Science* **1968**, *159* (3813), 390−396.
(22) Qhobosheane, M.; Santra, S.; Zhang, P.; Tan, W. H. *Analyst* **2001**, *126* (8), 1274−1278.
(23) Huang, X. H.; Jain, P. K.; El-Sayed, I. H.; El-Sayed, M. A. *Photochem. Photobiol.* **2006**, *82* (2), 412−417.
(24) Otsuka, H.; Nagasaki, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2003**, *55* (3), 403−419.
(25) West, J. L.; Halas, N. J. *Annu. Rev. Biomed. Eng.* **2003**, *5*, 285−292.
(26) Loo, C.; Hirsch, L.; Lee, M. H.; Chang, E.; West, J.; Halas, N.; Drezek, R. *Opt. Lett.* **2005**, *30* (9), 1012−1014.
(27) Schiffelers, R. M.; Ansari, A.; Xu, J.; Zhou, Q.; Tang, Q. Q.; Storm, G.; Molema, G.; Lu, P. Y.; Scaria, P. V.; Woodle, M. C. *Nucleic Acids Res.* **2004**, *32* (19), e149.
(28) Li, J.; Zheng, W. C.; Kwon, A. H.; Lu, Y. *Nucleic Acids Res.* **2000**, *28* (2), 481−488.

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