STUDIES AT THE NANOPARTICLE-BIOMOLECULAR INTERFACE AND BEYOND

BY

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DISSERTATION

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ABSTRACT

Gold nanoparticles (Au NPs) have attracted much interest in biological applications due to their excellent optoelectronic properties and ease of surface functionalization. However, while Au NPs are positioned to revolutionize nanotechnology based biomedical applications, our fundamental understanding at the nanoparticle-biomolecular interface and its resultant impact on cells is still limited. In particular, analysis of the spatial arrangement of nanoparticle’s surface ligands using scanning tunneling microscopy is a highly controversial topic. Addition of nanoparticles to cell culture media was shown to result in a hard and soft protein corona formation, which acted to mitigate/reduce the proposed chemical capabilities of nanoparticles. More critically but even less well understood is the precise orientation of proteins upon adsorption as well as the possible change in protein’s conformation, which can alter the protein’s intrinsic function. This dissertation will thus focus on developing our understanding at this interface, by probing the chemistries at this nanoparticle-biomolecular interface and subsequently, how the Au NPs influence cellular responses.

The question of spatial location of different ligands on nanoparticle surfaces with diameters less than 100 nm is an important one that is difficult to quantitatively address. To investigate the spatial arrangement of biomolecules on Au NPs, the surface of 20, 50, and 90 nm Au NPs were functionalized with two different lipids, both single and mixed, using two different surface chemical procedures utilizing electrostatic or hydrophobic interactions. Mass spectrometry supported the presence of both lipids in the mixed-lipid systems on nanoparticles, and it was observed that the surface chemistry of Au NPs influenced the relative ratios of mixed
lipids incorporated. Electron microscopy evidence showed domain sizes for one lipid apparently a quarter to a half the projected diameter for 50 and 90 nm particles; but for 20 nm particles, there is no evidence for the existence of patches of the two lipids.

To study the potential use of Au NPs to limit α-synuclein (α-syn) misfolding, the binding and orientation of α-syn on anionic and cationic Au NPs were investigated. On anionic Au NPs, α-syn was determined to interact with 20 and 90 nm Au NPs via multilayered adsorption, consisting of a strong electrostatic interaction between α-syn and Au NPs in the hard corona and a weaker noncovalent protein–protein interaction in the soft corona. On cationic PAH Au NPs, titration of α-syn into cationic Au NP at >2000 α-syn/cationic Au NP caused the flocculation and sedimentation of α-syn coated PAH Au NPs. The orientation of α-syn onto Au NPs was studied using protease digestion method, revealing that α-syn absorbs onto anionic Au NPs via its N-terminus while on cationic Au NPs, a random orientation of α-syn was adopted. Comparison of the digestion pattern of α-syn on both Au NP with respect to free α-syn reveal an increase in the release of peptides from the N-terminus (amino acid 1–23, lysine position 10) and a decreased number of peptides in the non-amyloid component region (amino acid 59–97, lysine position 80) when adsorbed onto Au NPs, suggesting that the adsorption and binding orientation of α-syn depends on the surface charge of Au NPs.

The aggregation of Au NPs in cell media is a common phenomenon that can influence NP-cell interactions. This interaction can be more precisely controlled by the formation of a protein corona on Au NPs before introduction into a high salt media. Cell viability assays showed that non-aggregated Au NPs were less toxic than their aggregated counterparts in human dermal fibroblast (HDF) cells. Fluorescence confocal imaging demonstrated that cellular F-actin fiber formation was less disrupted with non-aggregated Au NPs.
Differently functionalized Au NPs were found to induce different cellular responses when incubated with cells in vitro. Darkfield microscopy demonstrates that both prostate cancer cells (PC3) and HDF cells can “vacuum” Au NPs from the surface. Mean cumulative square distance of cells shows that PC3 migration decreases in the presence of Au NPs while for HDF, migration is dependent on the surface charge and shape of Au NPs. Preliminary investigations on the global impact of Au NPs with cells, based on gene expression analysis, demonstrated that genes related to cell proliferation were up-regulated while genes related to metabolism were down-regulated in HDF. In contrast, gene expression changes in PC3 were observed to strongly depend on surface functionalization of Au NPs, suggesting that Au NPs impact PC3 at a more fundamental molecular level.
Dedicated to my future wife, Hiu Fung

‘Marriage is work…’

Ben Affleck

Oscar Best Picture speech, 2013

‘You are a lot of work.’

Jennifer Garner

SNL monologue, 2013
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CHAPTER 1

An Introduction to Gold Nanoparticles*

1.1. Gold: From Bulk to Nano

Gold, as a noble metal, has been mined and used since the early ages and throughout the world. The earliest well-dated gold artifacts have been found in the Varna Necropolis (4600–4200 BC) in Bulgaria.¹ Gold has been described in Egyptian hieroglyphs from as early as 2600 BC.² The earliest documented use of gold for decorative purposes in China dates back to the Shang dynasty (~1500–1050 BC).³ The Metamorphoses (Book XI, AD 8), told tales of Midas, the king with the touch of gold.⁴ In Naturalis Historia (Book XXXIII and XXXIV, AD 77–79), Pliny the Elder described mining methods used in Rome for the extraction of gold and the correct uses of gold based on gender and class.⁵ Gold has shaped much of mankind’s history and even till today, still holds great importance and value: gold medals or trophies are still given to the champions of competitions as a symbol of achievement. While the use of gold in the past has been mostly in ornaments and trade, applications of gold have expanded in modern times. In its bulk form, gold is still used throughout the world as an investment to hedge against economic disruptions. Gold thin films are used in office windows to reflect infrared light while transmit visible light, thereby efficiently regulating temperature within the building.⁶ The high conductivity and high corrosion resistance of gold allows for its applications where good

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connections are crucial such as in USB cable, artificial satellites and aircraft engines. Molecular gold compounds are used in the treatment of arthritis.⁷

There is even greater value in gold on the nano-scale. The number of publications on gold nanotechnology has grown exponential since 1990, from ~20 to ~12,000 in 2012 (Figure 1.1). Over the years, the wet chemical synthesis method to make gold nanoparticles (Au NPs) have developed extensively, such that it is now possible to make Au NPs with excellent control over size and shape.⁸ This has led to much work focusing on understanding the surface chemistry of Au NPs, for its interaction with molecular targets or for self-assembly.⁹–¹¹ Further understanding of Au NPs’ plasmonic properties has led to observations of extremely large field enhancements, huge light scattering and absorption and photothermal capabilities.¹²–¹⁴ All the understanding has cumulated in numerous applications: Au NPs are being explored as vesicles for drug and gene delivery for cancer and other diseases, as chemical sensing agents, as catalyst, and in imaging platforms.¹⁵–²¹

1.2. Properties of Gold Nanoparticles

Nanoscale particles, or nanoparticles, are defined as particles with dimensions on the nanometer scale (1–100 nm). At this size, Au NPs exhibit properties that are fundamentally different from bulk gold. Under visible light, instead of the yellow color of bulk gold, Au NPs exhibit shape-dependent optical spectra when dispersed in solution, ranging from red for nanospheres, to green and brown as the aspect ratio increases (Figure 1.2). This interesting optical property is due to Au NP’s unique interaction with light, and is attributed to the collective oscillations of conduction band electrons in a metal when the metal particle size approaches the
Figure 1.1. Exponential growth in the number of publications on gold nanotechnology over the past two decades. Data was obtained via SciFinder, topic search for publications in gold nanotechnology 19 July 2013: “ts = (gold*) and ts = (nano*)”.
Figure 1.2. a) Normalized UV-vis spectra of short gold nanorods with aspect ratios A) $1.4 \pm 0.3$, B) $1.8 \pm 0.5$, C) $2.3 \pm 0.6$, D) $2.6 \pm 0.4$ and E) $2.8 \pm 0.4$. TEM images of gold nanorods corresponding to the spectra are shown on the right. All scale bars are 100 nm. b) Photograph of gold nanorods as their aspect ratio increases from left to right.
electron mean free path length (~10–100 nm). At specific wavelengths of visible light, the oscillating electromagnetic field is in resonance with the free electrons in Au NPs, which cause a collective oscillation of the electrons with respect to their positive metallic lattice. This phenomenon is termed as localized surface plasmon resonance (LSPR) and the reemitted electric field decays radiatively via light scattering or nonradiatively via conversion to heat. The resonance conditions can be determined from absorption and scattering spectroscopy. Gold nanospheres (isotropic) show one absorption maxima in water at ~520 nm, while gold nanorods (anisotropic) display two absorption maxima, one for the short axis (transverse band) at ~520 nm and the other for the long axis (longitudinal band) which is tunable from ~700–900 nm (Figure 1.2a). As the aspect ratio of gold nanorods increases, the longitudinal band extends out into the far-IR region past 1500 nm. The elastic light scattering of Au NPs makes them visible at the single-particle level in darkfield optical microscopy, suitable for in vivo tracking.

The surface chemistry of Au NPs can be easily tuned and altered depending on function. In general, surface chemistry of Au NPs can be altered via electrostatic or covalent interactions. Citrate capped gold nanospheres and cetyltrimethylammonium bromide (CTAB) bilayer coated gold nanorods can be polyelectrolyte coated in a layer-by-layer fashion. This method affords a means to change the surface charge of the Au NPs as well as the ability to confer additional functionalities to the Au NPs. For example, Au NPs coated with poly(allylamine hydrochloride) (PAH) or poly(acrylic acid) (PAA) can be used in subsequent carbodiimide chemistry for the conjugation of small molecules, proteins and antibodies. Thiolated molecules changes the surface chemistry of Au NPs by displacing the weaker bound citrate or CTAB ligands for the stronger gold-sulfur covalent bonds. Thiolated methoxypolyethylene glycol (mPEG) can be used to displace CTAB on gold nanorods to reduce its cytotoxicity and improve its
bioavailability. The use of bifunctional molecules such as mercaptopropionic acid or thiol-PEG-amine not only allows for functional modifications, but also helps improve the stability of Au NPs against aggregation.

1.3. Wet Chemical Synthesis of Gold Nanoparticles

The boiling citrate method is the most widely used method to synthesize gold nanospheres of 12–40 nm. First reported by Turkevich et al. in 1951, the synthesis only requires three starting materials, gold(III) chloride trihydrate (HAuCl₄), sodium citrate and water. When the mixture is brought to boil, sodium citrate reduces Au³⁺ to Au⁰, forming gold nanospheres which are stabilized by citrate ions as ligands. By changing the sodium citrate concentration, the size of gold nanospheres can be easily tuned. More recently, it was discovered that sodium citrate also acts to regulate the solution’s pH and depending on the amount added, can push the formation of gold nanospheres either through a nucleation-growth pathway or a nucleation-aggregation-smoothing pathway (where small Au NPs first formed, which then aggregated into larger particles and subsequently smoothed out). Larger gold nanospheres (>40 nm) cannot be produced by the boiling citrate method, beyond which the nanoparticles tend to become prolate spheriod instead of spheres. Highly monodisperse gold nanospheres (50–175 nm) can be made by a seed-mediated synthesis using hydroquinone as the reducing agent.

The Murphy group, along with others, has developed two general synthetic methods to prepare short and long gold nanorods with relatively high yield, excellent shape monodispersity and reasonably tight control over the dimensions of the particles (Figure 1.3). In both methods, a seed-mediated synthesis is utilized, where HAuCl₄ is reduced in the presence of the
Figure 1.3. Synthesis of short and long gold nanorods. Ascorbic acid reduces $\text{Au}^{3+}$ to $\text{Au}^{+}$, changing the gold solution from yellow to colorless. $\text{AgNO}_3$ controls the aspect ratio of short gold nanorods.
strong reducing agent sodium borohydride (NaBH₄). The isotropic gold seeds made are ~1.5–4 nm in diameter. In the short gold nanorods synthesis, these seeds are added to a growth solution containing more gold salt, cetyltrimethylammonium bromide (CTAB), a weaker reducing agent (ascorbic acid) and silver nitrate (AgNO₃), which acts as an impurity. Here, ascorbic acid only reduces Au³⁺ to Au⁺, allowing for anisotropic growth as Au⁺ is further reduced to Au⁰ on the nanoparticle surface over a longer time period. The amount of AgNO₃ added dictates the final aspect ratio of the nanorods. The amount of Ag in the nanorods is enough for a few monolayers of Ag on the surface of the gold core and XPS data suggest that Ag is preferentially localized on the surface.⁴¹,⁴² Gold nanorods made using this method are tunable in the range of 12 – 20 nm in diameter and 20 – 100 nm in length, covering aspect ratios up to 5.

Long gold nanorods are made using a three step synthesis method starting from gold seeds.²⁶ In this method, no AgNO₃ is added, but the timing of reaction in each step is adjusted to vary the aspect ratio (Figure 1.3). For example, for the synthesis of long gold nanorods with aspect ratio 14, seeds solution is added to the first growth solution, mixed and allowed to incubate for 15 sec, following which an aliquot is taken and mixed with the second growth solution. After an incubation of 30 sec, another aliquot is taken and added to the third growth solution and stored overnight. Gold nanorods with aspect ratio 10–20 can be made using this method.

1.4. The Promise of Gold Nanoparticles

Because of their excellent optoelectronic properties, Au NPs are used in many biological applications. For example, the surface plasmon of Au NPs can be utilized to absorb electromagnetic radiation, which results in the generation of highly localized heat.⁴³ By exciting
Au NPs with pulsed lasers, enough heat can be generated to disrupt cells and bacteria, or even release drug molecules trapped on Au NP surfaces. U87 cells can be specifically targeted from a mixture also containing MCF7 when Au NPs are functionalized with RDG peptides, which can subsequently be killed using pulsed laser. The enhanced Raman can be used for chemical sensing. By functionalizing Au NPs with fluorescently labeled nuclear localization signal peptides, progenitor and differentiated human neuroblastoma cells can be distinguished using surface enhanced Raman spectroscopy (SERS). The light scattering and absorption of light by Au NPs have been used for in vivo and in vitro imaging. Because of their nano-scaled size, when functionalized with multiple targeting groups Au NP conjugates show increased binding affinity as well as targeting selectivity for cells, and can be used in various delivery applications. In this sense, Au NPs have been used in gene regulation or delivery, or if coupled with the enhanced permeability and retention (EPR) effect, Au NPs can accumulate selectivity at tumor sites to exert their therapeutic effects.

1.5. Issues in Biological Applications: Protein Corona Formation

While the potential of Au NPs in biological applications is high, the understanding of the interfacial chemistry between nanoparticles and biomolecules is still little known (Figure 1.4). In this aspect, the protein adsorption event on nanoparticles has only been recently appreciated and might even be critical in helping understand why some applications works but others do not.

Nanoparticles when introduced into biological systems either in vitro or in vivo, will first encounter serum proteins in the cell media or the blood plasma. Serum proteins in cell media or in blood plasma contain more than 2,000 proteins, which consist of a combination of the subproteomes derived from different tissues. Proteins are responsible for the biological
**Figure 1.4.** Potential nanoparticle-biomolecular interactions. Serum proteins can coat nanoparticles to mask underlying functionalities and create a hard and soft protein corona. The orientation upon adsorption of proteins on nanoparticles may influence protein’s function and may alter conformation.
processes in cells, and collectively have a large range of isoelectric point (pI), hydrophobicity, affinities, structures and size, all of which contribute to their vastly different functions and interactions. Proteomic analysis suggested an estimate of at least ~10,000 different proteins in the U2OS (human osteosarcoma) cell line, with their abundance varying widely on seven orders of magnitude up to 20,000,000 copies. Low-copy-number proteins (less than 1000 molecules/cell) also play an important role in many cell functions.

Any colloidal material introduced into this complex system will undoubtedly be covered by proteins. Indeed, this was observed in work done on biomaterials (micron-sized or larger) for medical applications, where upon the introduction into biological environment, are immediately be covered by proteins, the composition of which changes over time. In the case for nanoparticles, the high surface to volume ratio, coupled with the potentially high surface energy of a curved surface, allows for an amplified protein adsorption scenario when compared to medical devices (pacemaker, stent, hip replacement, etc.). In addition, in contrast to devices which are usually stationary, nanoparticles can access almost every organ. There is thus growing consensus that cellular response to nanoparticles in a biological medium may be due to the absorbed biomolecule layer and not the material itself. For example, it was also shown more recently that the targeting ability of transferrin bound nanoparticles is lost when these nanoparticles are placed in a biological environment, and it is believed that the surface functionalization is masked due to the serum protein adsorption onto nanoparticles. As such, understanding the nanoparticle-biomolecular interface is thus a matter of high importance and urgency: low-copy-number proteins may be rendered less bioavailable when trapped on nanoparticles, throwing cellular pathways into disarray. Alternatively, overexpressed/misfolded
proteins can be sequestered onto nanoparticles and removed, returning the cells to their normal homeostasis.\textsuperscript{66,67}

The protein corona is used to describe the proteins absorbed onto nanoparticles. It is now known that a plethora of proteins exist in the protein corona, which bind due to numerous non-specific interactions with nanoparticles such as electrostatic interactions, hydrophobic interactions and avidity (protein-protein interactions). The wide array of interactions also means that proteins association to nanoparticles comprise of a large range of affinities for nanoparticle surface. In general, the protein corona can be characterized comprising of a hard and soft layer: the hard inner corona consist of strongly associated proteins and the soft outer corona consisting of weakly bound proteins in rapid exchange with free proteins in solution.\textsuperscript{68} Recent evidence had also shown that the hard protein corona is also susceptible to changes, albeit more slowly.\textsuperscript{69}

A more pertinent issue that arises from protein adsorption is the possible change in protein conformation when bound as well as its binding orientation.\textsuperscript{70} The native conformation of proteins frequently consist of $\alpha$-helix and $\beta$-sheets, which are held together in their tertiary structure by hydrogen bonding, van der Waals, electrostatic and hydrophobic interactions between the amino acids.\textsuperscript{71} Upon adsorption, the interaction of proteins with nanoparticle’s surface can alter some of its intrinsic interactions, both on the protein’s surface or in its hydrophobic core, thereby disrupting protein function. Such interactions may be energetically favorable: the decrease in entropy upon protein adsorption to nanoparticles is more than compensated by the increase in entropy of water release.\textsuperscript{72} Using circular dichroism, conformational changes of proteins have been detected on nanoparticles.\textsuperscript{73} Protein structural changes have also been found to be dependent on the nanoparticle structure, size and degree of saturation.\textsuperscript{74–76} Nanoparticles can also induce a larger scale protein folding change with amyloid
proteins. Standard chemical techniques are now being used to explore protein-nanoparticle interactions. In terms of protein orientation, NMR has been used to identify ubiquitin’s binding site to Au NPs. MALDI-MS had also been used to study the orientation of proteins on silica nanoparticles, which showed that nanoparticle size and protein structure influenced the protein’s orientation. Both the structural change and binding orientation of proteins onto nanoparticles can be potentially problematic as a loss of function or toxicity can be induced upon nanoparticle adsorption, which would limited the use of nanoparticles in biological applications.

1.6. Issues in Biological Applications: Toxicity and Cellular Changes

Many investigations of Au NP interactions with cells in vitro has thus focused on the cellular toxicity induced by Au NPs. It is now known that while the Au NP core is non-toxic, toxicity can be induced by the ions, capping agents and biomolecular ligands used to make and coat Au NPs. Therefore, extra care must be taken to ensure that excess free ligands are removed before any toxicity studies: incomplete separation and removal of free ligands can induce a stronger toxicity response than from the nanoparticles. CTAB is toxic at submicromolar concentrations and if not sufficiently removed, can induce toxicity in cells. Such adverse effects on cells can be overcome by overcoating Au NPs with polyelectrolytes and extensive purification.

However, beyond just toxicity, the interaction of Au NPs with cells can result in changes in cell structures. Fluorescent staining to cells showed that actin fibers as well as tubulin cytoskeleton were disrupted after Au NP uptake. The presence of Au NPs in cells also caused lysosomes to be enlarged and the number of autophagosomes increased, probably a result of a purge response. On a more macroscopic scale, cell adhesion of MC3T3-osteoblast cells to
integrin coated Au NP surfaces was influenced by the spacing of Au NPs. Au NPs can also alter the polymerization and mechanical properties of 2 and 3 dimensional collagen matrix, which cumulatively influenced cardiac fibroblasts response behavior.

Indeed, the diverse cellular response after Au NP incubation signifies that a more fundamental understanding and analysis of the interaction is required. It can be expected that while the toxic and non-toxic physical processes can be observed and monitored, many changes in the chemical and cellular pathways would go unnoticed. These alterations should by no means be dismissed as unimportant; most cellular networks and pathways are inter-linked, with proteins serving multiple roles and functions. Such detailed understanding can be obtained from gene expression analysis, which would allow for all the changes in the cell to be accounted for. For example, Au NPs’ size and shape can induce different cellular pathways related to cellular stress and toxicity. Nucleic acid functionalized Au NPs as well as citrate Au NPs did not induce significant gene expression changes in HeLa cells, while mercaptohexadecanoic acid functionalized Au NPs induced more changes than PEG coated Au NPs over the 84 genes probed in human keratinocyte cells. When human skin fibroblasts were incubated with carbon nanotubes and nano-onions, the gene responses were found to be dependent on nanoparticle concentration. While these studies illustrated that nanoparticles can have a global impact on cells, much of this impact is still relatively unknown.

1.7. Thesis Overview

The work described in this thesis is divided into two parts: 1) investigating the nano-bio interface through adsorption of biomolecules onto Au NPs, and developing methods to analyze biomolecule’s binding, orientation and conformation; and 2) understanding how the protein
corona interacts with cell media, and ultimately how Au NPs induce cellular phenotypic and genotypic changes. The remainder of the thesis will be structured with this outline in mind, with Chapter 2–4 focusing on part 1, and Chapter 5–7 focusing on part 2.

In Chapter 2, a procedure to synthesize mixed phospholipids coated Au NPs with different underlying surface chemistry is described. The goal of this work is to create lipid vesicle mimics of tunable sizes for chemical sensing and biomedical applications. Methods to analyze the phospholipid composition and arrangement are discussed, and it is found that the phase separation of mixed phospholipids on Au NPs is dependent on nanoparticle size. In Chapter 3, α-synuclein’s binding, orientation and conformation on negatively charged citrate Au NPs is studied, while in Chapter 4, α-synuclein’s orientation and conformation on positively charged poly(allylamine hydrochloride) coated Au NPs is studied. The aim of this work is to deduce possible protein folding changes as well as orientation preferences on Au NP with different surface functionalities, and thus investigate the use of Au NPs as a potential therapeutic agent for amyloid diseases. It was found that the underlying surface chemistry controls α-synuclein’s binding orientation and conformation, and the affinity of α-synuclein for Au NPs is a lot stronger than for lipid vesicles.

Chapter 5 discusses the non-specific adsorption of serum proteins on Au NPs during in vitro applications. Serum protein adsorption on Au NPs was shown to be influenced by Au NP’s surface chemistries, and the aggregation of Au NPs can be controlled by embracing the protein corona formation. The cellular uptake is shown to be influenced by the surface chemistry on Au NPs and its aggregation state. Chapter 6 details work on Au NP’s impact on cell mobility, where it is found that as cells transverse across a Au NP coated surface, they ‘vacuum’ up Au NPs which influences their migration. In Chapter 7 the short term effect of Au NP incubation on
cellular global gene expression is studied, and it is shown that the surface chemistries on Au NPs is the driving factor that results in different cellular pathways being up or down-regulated.
1.8. References


CHAPTER 2

Evidence for Patchy Lipid Layers on Gold Nanoparticle Surfaces*

2.1. Introduction

Much progress has been made in recent years in the synthesis and applications of gold nanoparticles (Au NPs). In addition to excellent control over size and shape, the ability to fine-tune the localized surface plasmon resonance of Au NPs underlies the potential for biological applications in imaging, sensing, drug delivery, and photothermal therapy. However, one continuing issue for biological applications of nanomaterials is that the reagents used in their synthesis can induce toxicity (in addition to possible negative effects of the nanoparticles themselves). In the case of Au NPs, a surfactant that is commonly used in their synthesis, cetyltrimethylammonium bromide (CTAB), is toxic at $10^{-7}$ M concentrations. In order to make Au NPs safe for biological applications, postsynthetic modification is usually required to alter the surface chemistry to make the particles more biocompatible (less prone to aggregation and less toxic). For example, polymers such as poly(ethylene glycol) (PEG) and polyelectrolytes such as poly(allylamine) hydrochloride (PAH) can be used to help stabilize Au NPs in aqueous solutions, as well as favor their uptake into cells. While this surface functionalization might solve the biocompatibility issue, recent studies have shown that polyelectrolyte-coated Au NPs

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can attract a large array of serum proteins to their surface, changing their $\zeta$ potentials and hence functionality.\textsuperscript{2} This is a significant issue if single or low copies of proteins, of which each has a distinct biological role, are trapped in this “protein corona”.\textsuperscript{6} As a means to overcome this problem, inspired by lipid vesicles that stabilize drug molecules and can be used as drug delivery systems,\textsuperscript{7} recent investigation has focused on using biomolecules such as phospholipids to passivate Au NP surfaces. In making Au NPs more “cell”-like, a 2-fold advantage is achieved: (i) biocompatible Au NPs with possibly reduced inflammatory response are produced and (ii) control over protein binding is possibly enabled.

Phospholipids are amphipathic in nature: the charge on the phosphate headgroups make them hydrophilic, while the acyl tail chains confer hydrophobicity. They form the major component of cell membranes and can generally be subdivided on the basis of their phosphate headgroups, of which phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) are the most abundant. For example, of the total lipid content in the brain, $20\text{--}30\%$ comprises PE, $10\text{--}25\%$ PC, and $5\text{--}10\%$ PS, and the remainder is made up of sphingolipids and sterols.\textsuperscript{8} In addition to variations in the headgroups, the saturation and length of the acyl tail chains modulate the fluidity and transition temperature of lipid layers.\textsuperscript{9} By modulating the lipid’s liquid-gel state, lipid layers can be deposited on flat and/or spherical silica surfaces to form a supported lipid bilayer membrane.\textsuperscript{10-15} While lipid coating on flat surfaces can be done with relative ease, pores form in the lipid layer when coated on nanostructured surfaces less than 22 nm in size.\textsuperscript{16,17} Regardless, it is agreed that the deposition of lipid layers on flat surfaces occurs first via the attachment of the vesicles to the support; the vesicle then spreads out and fuse with other patches to form a continuous lipid membrane.
Investigations on mixed lipid membranes on flat and structured surfaces revealed that lipid domains can result.\textsuperscript{18–22} The phase separation of lipids is a result of the fluidity, attraction, and repulsion behavior of the lipids, influenced by the acyl tail chain length,\textsuperscript{23} saturation of the acyl tail chains,\textsuperscript{24,25} variations in phosphate headgroups,\textsuperscript{26,27} and charge as introduced by ions, pH, and charged proteins.\textsuperscript{19,28,29} In particular, on a curved environment, lipid mixtures also show a strong tendency to phase-separate as a means to reduce lateral pressure and area density induced by the curvature.\textsuperscript{20–22} Lipids can coat nanoscale silica particles. When a single lipid is used, a homogeneous lipid bilayer forms on spherical silica particles.\textsuperscript{11,14} Mixed lipids have also been shown to form homogeneous layers on nanoscale silica, as revealed with cryoelectron microscopy and NMR and IR spectroscopy.\textsuperscript{12,30,31} Mixed lipid bilayers have been shown to be stable on polysilicon nanowires.\textsuperscript{32} However, the formation of lipid domains on nanosized silica particles has been little discussed. Recent work on lipid coating of Au NPs shows great promise to enable biocompatibility.\textsuperscript{33–36} Several methods now exist to favor formation of the lipid bilayer on Au NPs, which include use of a thiolated lipid for the strong sulfur–gold bond formation and a mixture of single- and double-chain fatty acid-type lipids to increase the packing density on the curved surface.\textsuperscript{35,37} By surface ligand exchange, Orendorff et al. showed that phospholipids can be used to displace CTAB on Au nanorods, albeit not completely.\textsuperscript{38} These phospholipid Au nanorod composites can be used to sequester lipophilic drug molecules into the bilayer, acting to stabilize these hydrophobic molecules in an aqueous environment.\textsuperscript{37} Other cargo such as RNA, DNA, and protein can potentially be attached to the surface of Au nanorods, allowing their use in targeted delivery.\textsuperscript{39} The situation for spherical Au NPs and lipids is not so straightforward. The chemistry changes with respect to NP size, due to the increasingly high surface curvature as the
NP size decreases. For example, 60 nm Au spheres can be encapsulated with lipids, while sub-4-nm spheres are usually found embedded in the bilayer of lipid vesicles.\textsuperscript{33–36}

Investigations of lipids with Au NPs thus far have focused on using lipids with one type of phosphate headgroup and do not truly mimic the highly varied lipid composition of cell membranes. Mixed-lipid-coated Au NPs are therefore a crucial next step in biocompatible NP chemistry, as they allow for the mimicry of cellular surfaces in terms of solvent-facing functional groups and charge density. In a broader sense, the generation of “patchy” NPs is of great fundamental interest for nanoscale assembly as well as in biomimetic systems.\textsuperscript{40–42} However, while proven to be theoretically feasible via molecular simulations,\textsuperscript{41,43,44} it has been very difficult to prove the existence of patches on colloidal nanoscale surfaces. Nevertheless, using scanning tunneling microscopy, Stellacci and co-workers have evidence that, for Au NPs of diameter $\sim 5$ nm, particular pairs of thiols form organized stripes (or “ripples”) that are sub-2-nm in width.\textsuperscript{45} Phase segregation of mixed thiols on Au NPs can also be probed by matrix-assisted laser desorption/ionization coupled with ion mobility-mass spectrometry as well as spectrophotometric titrations with fluorescent probes.\textsuperscript{46,47} These techniques, which require strong gold–sulfur binding and have been shown to work only on small Au NPs, are not feasible for lipid adsorption on Au NPs, as electrostatic interactions are usually the main attractive force. In this work, we have successfully coated mixed lipid layers on a variety of highly curved Au NP surfaces with diameters ranging from 20 to 90 nm. Lipid bilayers were formed on poly(allylamine) hydrochloride (PAH) coated Au NPs and “hybrid” lipid bilayers were formed on octadecanethiol-coated Au NPs, taking advantage of the lipid tail association with the hydrophobic self-assembled monolayer of the octadecanethiol.
Evidence of lipid coating was obtained through various spectroscopy techniques as well as transmission electron microscopy (TEM) imaging, which suggests that the mixed lipid phase-separates on nanoparticle surfaces and exists as lipid domains. Depending on the surface charge of the Au NP, the type of lipid that is preferentially adsorbed on the Au NP is also varied. These lipid-coated Au NPs showed increased stability against aggregation over a larger pH range as compared to their native NPs. To demonstrate the versatility of lipid-coated Au NPs, the lipid layer served as a base to coordinate other lipid–protein complexes.

2.2. Materials and Methods

2.2.1 Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium citrate tribasic dihydrate (Na₃Cit·2H₂O, ≥99%), hydroquinone (≥99%), 1-octadecanethiol (98%, C₁₈SH), poly-(allylamine hydrochloride) (PAH; MW 15 000 g/mol), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), monoclonal anti-biotin antibody (mouse), and OptiPrep density gradient medium were obtained from Sigma Aldrich and were used as received. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt) (biotin PE) in chloroform were obtained from Avanti Polar Lipids and were used as received. Osmium tetroxide (OsO₄, 4%, aqueous) was obtained from EMS and diluted to 0.1% before use. Ultrapure deionized water (17.9 MΩ, Barnstead NANOpure II) was used for all solution preparations. Glassware was cleaned with aqua regia and rinsed thoroughly before use.
2.2.2. Synthesis of Gold Nanoparticles

Au NPs of diameter 12 and 20 nm were synthesized via the boiling citrate method as previously described.48,49 Larger NPs (50 and 90 nm) were synthesized with 12 nm particles used as seeds.50 Briefly, 2 mL of a 1% (w/v) HAuCl4·3H2O solution was centrifuged at 17,000 relative centrifugal force (RCF) for 60 min after which the top 1 mL was obtained and added to 94 or 97.25 mL of ultrapure deionized water and 4 or 0.75 mL of 12 nm NPs for the resultant 50 or 90 nm particles, respectively. The solution was then stirred rapidly at room temperature and 0.22 mL of 1% trisodium citrate dihydrate was added, followed immediately by 1 mL of 0.03 M hydroquinone. The solution was allowed to stir overnight.

2.2.3. PAH Coating of Gold Nanoparticles

A 1 mL aliquot of as-made Au NPs was centrifuged and purified. The Au pellet was collected and resuspended in 1 mL of water. To this purified Au NPs pellet, 100 μL of NaCl (0.1 M) and 200 μL of PAH (10 mg/mL) were added simultaneously, and the solution was vortexed. The NPs were allowed to incubate overnight before being purified by centrifugation.

2.2.4. Preparation of Lipid Vesicles

For mixed lipids on Au NPs, a 1:1 weight ratio of POPS/LPC was used in all cases. Briefly, a total of 1 mg of lipid (0.5 mg of each POPS and LPC) in chloroform was dried under a stream of nitrogen. The lipid film was then further dried in vacuum for about 6 h, after which 20 mM HEPES buffer was added to give a final concentration of 2 mg/mL. The mixture was briefly sonicated to totally suspend the lipids, affording a clear colorless solution. Dynamic light scattering measurements of these vesicles gave an average hydrodynamic diameter of ∼90 nm.
2.2.5. Synthesis of Lipid-Coated Gold Nanoparticles

Au NPs were centrifuged to remove the excess ligands from the synthesis and redispersed to 0.5 mL in 20 mM HEPES buffer. For mixed lipid bilayers on Au NPs, 0.5 mL of the 1:1 POPS/LPC lipid solution from the above section was added to PAH coated Au NPs and mixed. For mixed lipid hybrid bilayer on Au NPs, 0.5 mL of the lipid solution was added to purified citrate capped NPs, followed by an appropriate amount of C$_{18}$SH (5 μg/mL ethanol). The mixture was incubated overnight at room temperature. The mixture was then centrifuged and the Au pellet was resuspended in HEPES buffer. Analysis of these composites was performed within 24 h.

2.2.6. Osmium Tetroxide Staining for Transmission Electron Microscopy Samples

A small amount of concentrated sample (8 μL) was dropped onto a TEM grid and allowed to sit for about 1 h. Excess sample was wicked off by use of Whatman filter paper. The grid was then floated on top of 0.1% OsO$_4$ stain (4% OsO$_4$ diluted with water) for 1−1.5 h. The grid was wicked dry and washed three times with water for 10 min each.

2.2.7. Quantification of Bound Lipids using Mass Spectrometry

Lipid-coated Au NPs were twice centrifuged to remove excess unbound lipids and concentrated down to a volume of 100 μL. The concentration of the Au NPs was determined by use of their known extinction coefficients from UV−visible spectroscopy. A known amount of either 1 M KCN or aqua regia was added to the solution to etch the Au NPs for about 2 h at room temperature. The colorless solution was then analyzed by HPLC/tandem mass spectrometry (MS/MS). The HPLC flow rate was set at 0.35 mL/min. HPLC mobile phases consisted of A (25
mM ammonium acetate in H2O) and B (MeOH). The gradient was: 0–0.5 min, 50% A; 5–16 min, 0% A; 16.5–25 min, 50% A. The autosampler was kept at 5 °C. The injection volume was 1 μL. The mass spectrometer was operated under both positive and negative electrospray ionization. The electrospray voltage was set to −4500 V in negative mode and 5500 V in positive mode, the heater was set at 600 °C, the curtain gas was 35, and GS1 and GS2 were 50, 50, respectively. Quantitative analysis was performed via multiple reaction monitoring (MRM), where m/z 496.4 and m/z 104.1 (positive mode for LPC) and m/z 760.5 and m/z 673.5 (negative mode for POPS) were monitored.

2.2.8. Conjugation of 12 nm Gold Nanoparticles to Larger Lipid-Coated Gold Nanoparticles with Protein Linkers

A 3:1 weight ratio of either POPS or LPC/biotin PE was used for the lipid mixture. A 1:2:1 weight ratio of POPS/LPC/biotin PE was used for the mixed lipid mixture. The lipid mixture was prepared following the method described above. Au NPs were incubated with the lipid mixture overnight and then purified. Au NPs of diameter 12 nm were centrifuged at 6,000 rcf for 30 min, and 10 μg of anti-biotin antibody (HEPES, pH 9) was added and the mixture was allowed to incubate for 1 h. The conjugated Au NPs were centrifuged at 6000 rcf for 30 min and added to the larger lipid-coated Au NPs, followed by incubation for 2 h at room temperature. The complexed Au NPs were then centrifuged at 500 rcf for 15 min, redispersed in water, and coated with PAH as described above. To separate bound from unbound 12 nm NPs, we found it necessary to employ a density gradient of OptiPrep (60% w/v iodixanol in water). Briefly, 0.2–1 mL solutions of varying densities (50%, 43.3%, 38.9%, 35.9%, 35.9%, 33.9%, 32.6%, 31.7%, 31.1%, and 30% iodixanol) were made by dilution with water. These solutions were then
layered in a 15 mL eppendorf tube, with the most dense solution at the bottom. The Au NPs solution was layered on top without disturbing the density gradient, and the tube was centrifuged at 500 rcf for 15 min. Larger lipid-coated Au NPs complexed with 12 nm NPs would be denser and travel down the density gradient, appearing as a red band. These were collected and purified twice via centrifugation before imaging by electron microscopy.

2.2.9. Instrumentation

Absorption spectra were taken on a Cary 500 scan UV–vis–near-IR spectrophotometer. Transmission electron microscopy data were obtained with a JEOL 2100 cryo electron microscope operating at 200 kV. Zeta potential and dynamic light scattering measurements were performed on a Brookhaven Zeta PALS instrument. LC/MS/MS analysis was performed in the Metabolomics Center at University of Illinois Urbana–Champaign (UIUC) with a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA) which is equipped with a 1200 Agilent LC. Analyst (version 1.5.1, Applied Biosystems) was used for data acquisition and processing. A Phenomenex (Torrance, CA) column [Kinetex 2.6 μm pentafluoropentyl (PFP) phase, 100 × 4.6 mm] was used for the separation.

2.3. Results and Discussion

2.3.1. Design and Synthesis of Lipid Bilayers or Hybrid Bilayers on Au NP Surfaces

The amphiphilic nature of lipids is due to their polar headgroup and the nonpolar fatty acid tails. Association of lipids with Au NPs can thus potentially occur either via electrostatic interactions of the heads with charged species on the Au NP surfaces or through hydrophobic interactions with the tails and hydrophobic species on the Au NP surfaces. Electrostatic interaction of the negatively charged headgroup of a lipid with positively charged
poly(allylamine hydrochloride) (PAH) coated Au NPs would thus give lipid bilayers on NPs in water (Scheme 2.1). In this design, the polar head of one lipid layer faces the cationic particle surface, and in order to sequester the hydrophobic tail from water, a second lipid layer associates with the Au NPs. Conversely, functionalizing the Au surface with an alkanethiol, 1-octadecanethiol (C₁₈SH), produces a hydrophobic surface that allows for interaction with the tail ends of lipid, which we term “hybrid” bilayers (since the bilayer is composed of the alkanethiol and a lipid, not two lipids).³³ In our investigations, lipids with two different phosphate headgroups were studied: a saturated single fatty acid chain compound, lysophosphatidylcholine (LPC), and a monounsaturated double fatty acid chain compound, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS). The phosphatidylcholine (PC) and phosphatidylserine (PS) headgroups were chosen because they are two of the four major types of lipids found in the human body.⁹

The choice of a 1:1 POPS/LPC lipid formulation was based on previous work with other types of nanomaterials. Fischlechner et al. had previously shown that a 1:1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/POPS mixture was ideal for the formation of a homogeneous lipid bilayer on PAH-terminated silica particles (3–8 μm).³¹ As expected, negatively charged POPS was attracted to the positively charged PAH through electrostatic interactions. Zwitterionic POPC was also found to associate with PAH, and it was postulated that hydrogen bonding occurs between the phosphate on POPC and the amine on PAH. We therefore surmised that a 1:1 ratio of two related lipids would be favorable for bilayer formation on Au NPs as well. However, to accommodate the huge increase in surface curvature for the 20–90 nm NPs compared to 3–8 μm silica particles, we substituted LPC for POPC, reducing the acyl tail chains from two to one. The single acyl tail reduces the steric hindrance of LPC, allowing it to
Scheme 2.1. Formation of Lipid Bilayers or Hybrid Bilayers on Au NP Surfaces

With PAH coated Au NPs (blue on gold), a lipid bilayer results. Addition of alkanethiol (C\textsubscript{18}SH, purple) to citrate-capped Au NPs results in the formation of hybrid bilayers on the Au NPs. The labels 1-6 refer to Au NPs with (1) a POPS bilayer, (2) a mixed POPS/LPC bilayer, (3) an LPC bilayer, (4) a POPS hybrid bilayer, (5) a mixed POPS/LPC hybrid bilayer, and (6) an LPC hybrid bilayer.
adopt a conical shape and conform onto a smaller NP (Scheme 2.2). The trimethylammonium headgroup also makes it similar to cetyltrimethylammonium bromide (CTAB), which is commonly used to make Au NPs.\(^5\) In fact, LPC is known to act as a detergent and forms micelles of size 5 nm.\(^6\) This potentially reduces the spatial requirement at the hydrophobic tail end, allowing for tighter packing on the highly curved surface of Au NPs. The other lipid in our construct, POPS, has its cylindrical shape imposed by the two acyl tail chains, which favor larger liposome bilayers due to its low intrinsic curvature compared to LPC (Scheme 2.2).\(^7\) In addition, POPS was specifically chosen for its low transition temperature (T\(_m\)) of 14 °C, allowing experiments to be performed at room temperature while maintaining the lipid layer in its fluid state.

In order to discover which combination of lipids would provide colloidal stability to Au NPs in organic solvent (presumably due to lipid monolayer formation), we layered an aqueous phase containing PAH-coated 20 nm Au NPs on top of a chloroform layer containing various lipids. The mixtures were vigorously mixed and allowed to stand overnight. Results showed that only when a mixture of 1:1 POPS/LPC was used were Au NPs transferred cleanly into the organic phase (Figure 2.1). This highlights the notion that the formation of a lipid layer on a highly curved surface is more favored when mixtures of lipids are used, possibly as a result of better packing and hence passivation of Au NP surface. This is consistent with results from lipid coating on silica particles. We were thus motivated to explore lipid bilayer coatings on Au NPs composed of either POPS or LPC alone, and a mixture of 1:1 POPS/LPC.
Scheme 2.2. Cartoon of a Micelle Made of LPC and a Liposome Made of POPS

LPC micelle is shown in gray and POPS liposome is shown in red. The single tail of LPC makes it difficult for LPC to fill all the volume of a bilayer while accommodating the area of the headgroup, resulting in a micelle structure instead of a liposome.
Figure 2.1. A photograph illustrating the distribution of Au NPs in water/chloroform mixtures in the presence of lipids. The top solvent layer is water; the bottom is chloroform. The red color indicates the phase in which the gold nanoparticles, originally coated with PAH, reside. Lipid identity in 1:1 ratios or as single components is indicated under each vial.
2.3.2. Characterization and Stability of Lipid Bilayers on Au NPs

Lipid bilayer formation was achieved, in principle, by incubating PAH-coated Au NPs with (1) POPS, (2) a 1:1 mixture of POPS/LPC, and (3) LPC (Scheme 2.1). To form the hybrid lipid layer, a ratio of 1 or 2 C18SH to surface Au atoms was chosen on the basis of recent X-ray crystallography, which showed that thiols bind to either 1 or 2 Au atoms on the surface of small Au NPs.55 Incubation of citrate-capped Au NPs in the presence of octadecanethiol (C18SH) with (4) POPS, (5) a 1:1 mixture of POPS/LPC, and (6) LPC should result in a hybrid bilayers on the gold surface, using the thiol as the bridging ligand to create hybrid bilayers (Scheme 2.1). Au NPs of various diameters (20–90 nm) were employed as substrates. These lipid-coated Au NPs were characterized with UV−vis spectroscopy, dynamic light scattering (DLS), and ζ potential measurements. In the remainder of the section, Au NPs will be referred to by their surface (1–6, Scheme 2.1) and their gold core diameter in nanometers, enclosed in brackets.

Ultraviolet−visible spectroscopy measurements of Au NPs showed a slight red shift in the wavelength maximum of ~2−3 nm after functionalization with lipids for most samples, consistent with an increase in the refractive index, suggesting lipid attachment (Figure 2.2).35 LPC alone on PAH-coated Au NPs that were 20 nm in diameter (Scheme 2.1; [3, 20 nm]) aggregated upon separation of free LPC and resuspension in HEPES buffer. Since LPC is only adsorbed to the PAH layer via hydrogen bonding if the results on silica surfaces apply, the lipid bilayer is easily desorbed, causing subsequent aggregation in water. The hybrid bilayers with POPS alone on 50 and 90 nm gold nanospheres (Scheme 2.1; [4, 50 nm] and [4, 90 nm]) showed blue shifts in the UV−vis rather than red shifts. These blue shifts were fairly reproducible and were observed only with POPS alone. While we are currently unsure of the significance of this blue shift, the shift does at least provide qualitative evidence for a new surface coating.
Figure 2.2. UV-vis spectra of Au NPs before (dotted lines) and after (solid lines) lipid functionalization, for three different gold core sizes. (a) Au NPs coated with PAH and then POPS, of diameter 20 nm (black), 50 nm (red) and 90 nm (blue). (b) Au NPs coated with PAH and then 1:1 POPS:LPC, of diameter 20 nm (black), 50 nm (red) and 90 nm (blue). (c) Au NPs coated with PAH and then LPC, of diameter 20 nm (black), 50 nm (red) and 90 nm (blue). (d) Au NPs coated with 1-octadecanethiol and POPS, of diameter 20 nm (black), 50 nm (red) and 90 nm (blue). (e) Au NPs coated with 1-octadecanethiol and 1:1 POPS:LPC, of diameter 20 nm (black), 50 nm (red) and 90 nm (blue). (f) Au NPs coated with 1-octadecanethiol and LPC, of diameter 20 nm (black), 50 nm (red) and 90 nm (blue).
An increase of about 3–20 nm in the hydrodynamic size of the Au NPs was detected after lipid coating via dynamic light scattering (Figure 2.3), consistent with the addition of nanoscale organic layers. In all cases the electrostatic PAH-enabled coating (Scheme 2.1) led to larger apparent particle diameters than the alkanethiol hydrophobic approach (Scheme 2.1), possibly suggesting the formation of lipid multilayers. No evidence for bulk Au NP aggregation was apparent by either UV–vis or light scattering measurements. Zeta potential measurements showed the change in NP surface charge after lipid coating (Figure 2.4a, b). For example, the ζ potential of 20 nm PAH-coated Au NPs changed from $+55.92 \pm 2.75$ mV to $-87.61 \pm 4.92$ mV after POPS coating, to $-92.66 \pm 1.60$ mV after POPS/LPC coating, and to $+20.51 \pm 1.36$ mV after LPC coating. Similar changes were seen for hybrid bilayer Au NPs: 20 nm citrate-capped Au NPs changed from $-36.46 \pm 1.36$ mV to $-103.8 \pm 2.85$, $-70.99 \pm 1.59$, and $-18.9 \pm 2.74$ mV after coating with POPS alone, POPS/LPC, and LPC alone, respectively. The presence of negatively charged POPS in the bilayer lowered the ζ potential to a negative value, while zwitterionic LPC reduced the inherent surface charge.

As the 1:1 POPS/LPC surface coatings most closely mimic the heterogeneity found in the cell membrane (compared to pure POPS or LPC coatings), we examined Au NPs with these coatings made by the two methods (Scheme 2.1). Further evidence for a mixed lipid coating on Au NPs comes from the ζ potential measurements with respect to pH (Figure 2.4). Results showed that 1:1 POPS/LPC-coated Au NPs were more stable to aggregation and exhibited different ζ potential changes as a function of pH compared to native Au NPs. PAH-coated Au NPs maintained a positive ζ potential above pH 10 and citrate-capped Au NPs aggregated immediately below pH 8, limiting the range of measurements for these samples. In contrast, both types of mixed-lipid-coated Au NPs maintained a negative ζ potential until pH 3.9. In a mixed
Figure 2.3. Dynamic light scattering data for Au NPs after 1:1 POPS:LPC lipid coating for three different gold core diameters (20 nm, 50 nm, 90 nm): initial citrate-capped (black), 1-octadecanethiol plus lipid (red), PAH-coated (blue) and PAH-coated plus lipid (green). All measurements were made in HEPES buffer at pH 7.
Figure 2.4. Zeta potential and light-scattering measurements to qualitatively establish surface coverage changes upon lipid functionalization. (a) Zeta potential measurements of 20 nm diameter Au NPs with PAH and lipid layers 1, 2 and 3 (Scheme 1). (b) Zeta potential measurements of 20 nm octadecanethiol-capped Au NPs with lipid layers 4, 5 and 6 (Scheme 1). (c) Zeta potential variations with respect to pH for 20 nm (black), 50 nm (blue) and 90 nm (red) Au NPs with 1:1 POPS:LPC with an initial coating of PAH. The behavior of native PAH Au NPs (20 nm) are given in black dotted lines. (d) Zeta potential variations with respect to pH for 20 nm (black), 50 nm (blue) and 90 nm (red) Au NPs with 1:1 POPS:LPC with an initial coating of 1-octadecanethiol. The behavior of native citrate Au NPs (20 nm) are given in black dotted lines. (e) Hydrodynamic diameter changes with respect to pH for 20 nm (black), 50 nm (blue) and 90 nm (red) Au NPs with 1:1 POPS:LPC with an initial coating of PAH. The data for native PAH Au NPs (20 nm) are given in dotted lines. (f) Hydrodynamic diameter changes with respect to pH for 20 nm (black), 50 nm (blue) and 90 nm (red) Au NPs with 1:1 POPS:LPC with an initial coating of 1-octadecanethiol. The data for native citrate Au NPs (20, 50, 90 nm) are given in dotted lines.
PC and PS vesicle, the pKa of the carboxyl group was 3.6 ± 0.1 and the pKa of the amino group was 9.8 ± 0.1. The pH change at 3.9 is very close to the carboxyl group pKa, suggesting the presence of a PS and PC layer.

Mixed-lipid-coated Au NPs, for 50 and 90 nm diameters, showed increased resistance to aggregation with respect to pH changes as compared to the native PAH or citrate Au NPs (Figure 2.4e,f). The native PAH-coated Au NPs were unstable at pH > 7, while native citrate-capped Au NPs aggregated below pH 7. Au NPs bearing the nominal 1:1 POPS/LPC, for 50 and 90 nm diameters, maintained their hydrodynamic sizes throughout pH changes and did not aggregate. The 20 nm Au NPs bearing 1:1 POPS/LPC were stable above pH 7 but tended to aggregate below pH 7. We postulate that the high surface curvature of these smaller particles favors the homogeneous distribution of the mixed lipids (vide infra). When mixed lipids distribute homogeneously on a curved surface, the difference in the stericz of LPC and POPS leaves much space in the lipid layer, decreases the packing density, allowing small ions to penetrate into the hydrophobic lipid layer and hence destabilize it, causing the aggregation. On larger 50 and 90 nm NPs, lipid domain formation is favored, thus allowing better packing and conferring greater stability to these lipid NP composites.

Mass spectrometry was used to quantify the number of lipid molecules associated per Au NP. Table 2.1 shows the number of POPS and LPC molecules per Au NP of diameters 20 and 90 nm. While a ratio of 1:1 POPS/LPC was incubated with the Au NPs, a different adsorbed ratio was found for the hybrid Au NPs. For example, on 20 nm hybrid Au NPs, a 13-fold excess of LPC over POPS was detected. Similarly, on 90 nm hybrid Au NPs, 28 times more LPC molecules were associated with the gold nanoparticles as compared to POPS molecules. A distinctly different trend was observed for the mixed lipids on PAH-coated Au NPs. For mixed
Table 2.1. Number of Lipid Molecules Detected per Au NP by Use of LC/MS/MS and Their Respective Ratios

<table>
<thead>
<tr>
<th>Au NP</th>
<th>POPS</th>
<th>LPC</th>
<th>Ratio (LPC/POPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 nm Au NPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hybrid (with C$_{18}$SH)</td>
<td>36 ± 4</td>
<td>460 ± 190</td>
<td>13</td>
</tr>
<tr>
<td>bilayer (on PAH)</td>
<td>8400 ± 900</td>
<td>8300 ± 500</td>
<td>0.99</td>
</tr>
<tr>
<td>90 nm Au NPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hybrid (with C$_{18}$SH)</td>
<td>2600 ± 400</td>
<td>73000 ± 18000</td>
<td>28</td>
</tr>
<tr>
<td>bilayer (on PAH)</td>
<td>48000 ± 7500</td>
<td>89000 ± 41000</td>
<td>1.85</td>
</tr>
</tbody>
</table>

*The lipids were incubated with the Au NPs at a ratio of 1:1 (POPS:LPC) before purification. For reference, ~1300 or 2600 total lipids would constitute a monolayer or bilayer around 20 nm Au NPs, respectively, and ~25,000 or 50,000 total lipids would constitute a monolayer or bilayer around 90 nm Au NPs, respectively.*
lips on 20 nm PAH Au NPs, the nominal 1:1 ratio of LPC/POPS was maintained (8400 ± 900 POPS versus 8300 ± 500 LPC molecules), while on 90 nm PAH Au NPs, a 1.85 LPC/POPS ratio was measured. These results demonstrate that the surface coating on Au NPs can influence the adsorption of lipids, although the imbalance of POPS and LPC is surprising for the thiol-modified, hybrid Au NPs. The zwitterionic charges as well as the single acyl tail chain of LPC could allow for tighter packing in hybrid Au NPs than POPS (negatively charged, more repulsion between POPS molecules), resulting in a higher ratio of LPC incorporated than POPS. On the other hand, positively charged PAH on Au NPs could strongly attract POPS molecules via electrostatic interactions as compared to LPC, resulting in increased POPS adsorption.

Large differences in the total lipid content between hybrid lipid on Au NPs and lipid bilayer on PAH Au NPs were also measured. On 20 nm Au NP, 36 ± 4 POPS and 460 ± 190 LPC molecules were in the hybrid lipid layer, compared to 8400 ± 900 POPS and 8300 ± 500 LPC molecules in the bilayer on PAH-coated Au NPs. A similarly large difference was observed for POPS on 90 nm Au NPs (Table 2.1). The much higher lipid counts on PAH-coated Au NPs implies lipid multilayer formation on PAH-coated Au NP surfaces; the differences are too great to be attributed to tighter packing of the lipids on one type of NP versus another. Simple footprint estimates of 1 nm² for each lipid suggest that 20 nm Au NPs should accommodate 1300 lipids/Au NP if the lipids formed a monolayer, or at least 2600 lipids/Au NP if the lipids formed a bilayer. Clearly, the total lipid content of the hybrid bilayers is far short of this footprint estimate, while for the PAH-coated 20 nm Au NPs, the detected lipid content is far higher than a simple mono- or bilayer. For 90 nm Au NPs, similar estimates give 25 000 lipids for a monolayer or 50 000 for a bilayer; the experimental mass spectrometry data are within a factor of 2 of the estimated coverage.
Interestingly, the hybrid-lipid-coated Au NPs using C_{18}SH were resistant to cyanide etching, even though there are fewer lipid molecules per nanoparticle than the PAH-coated gold nanoparticles. These hybrid NPs were not etched with 1000 μL of 1 M KCN as compared to citrate-capped NPs, which can be etched with as little as 20 μL for the same volume and concentration of NPs. The hybrid NPs were stable in the presence of KCN for up to a week, demonstrating the extent of thiol passivation-induced protection of Au NPs surface. This is similar to results published by Sitaula et al.,^{57} who showed that a 6-fold excess of 1-decanethiol to surface Au atoms can be used to stabilize Au NPs synthesized with a phosphatidylcholine lipid against cyanide etching. It is worth pointing out that, in our case, a lower concentration of alkanethiol is used to yield the same results. However, we have no information about the distribution of C_{18}SH and lipid in the inner and outer leaflet.

The visualization of lipid bilayers on Au NPs was performed by TEM imaging with a positive stain, osmium tetroxide (OsO_4). OsO_4 works by reacting preferentially with cis double bonds in unsaturated lipids to form a cyclic compound, allowing the lipid to be visible when viewed under the TEM.^{58,59} Given enough time and at the right temperature, OsO_4 can oxidize even alkanes in a [3 + 2] addition of a C−H bond to two oxo ligands of OsO_4.^{60,61} By precisely controlling the staining time at room temperature, we were able to preferentially stain only POPS while LPC (saturated) remained unstained and unobservable.^{62} Electron micrographs of POPS-coated Au NPs 1 and 4, stained with OsO_4, are shown in Figures 2.5 and 2.6 for 20 and 90 nm diameter particles. The expected thickness of a lipid bilayer is about 5 nm,^{9} while the measured organic layer thickness for 1 and 4 ranges from 2.2 to 3.3 nm and 2.3 to 4.6 nm for 20 nm NPs, and 3.1 to 9.0 nm and 3.3 to 6.2 nm for 90 nm NPs, respectively. As the drying and processing steps can greatly alter apparent organic layer thicknesses, we conclude that the data at the
Figure 2.5. Stained TEM images of PAH-coated Au NPs with POPS ([1, 20 nm] and [1, 90 nm]). Scale bars = 20 nm.
Figure 2.6. Stained TEM images of C$_{18}$SH coated Au NPs with POPS (4-20nm and 4-90nm).

Scale bars = 20 nm.
minimum support a continuous bilayer of POPS around these particles. In some cases, multilayered lipids on Au NPs were observed at a low fraction, as seen from the striation in the organic layer (Figure 2.7a). While variations in organic layer thickness were also seen on 20 nm Au NPs, this is not as obvious (Figure 2.8). The lack of a double bond in LPC renders LPC-functionalized particles less likely to be stained with this method. In order to demonstrate complete passivation of LPC-coated Au NPs 3 and 6, cryo TEM was performed (Figure 2.9). Cryo TEM images showed a continuous layer of organic material surrounding the Au NPs, suggesting that a homogeneous layer of LPC exists on Au NPs 3 and 6.

2.3.3. Formation of Lipid Domains on Au NP Surfaces

The presence of single types of lipids on Au NPs was confirmed by spectroscopy, light scattering, and the appropriate electron microscopic techniques. For Au NPs with the nominal 1:1 POPS/LPC coatings, the most interesting question is, do these mixed lipids form domains on the NP surface? The preferential staining of OsO₄ for unsaturated lipids allows for the visualization of POPS domains on Au NP surfaces. If the amount of surface-bound POPS is large enough, and its distribution about the NP surface was random, we would expect to observe a (faint) complete organic layer around the particle. Alternatively, if domains of POPS were present, NPs with patchy stained organics would be observed. The term patch, instead of domain, is used here to denote the distribution of lipids due to the diffusive nature of OsO₄; that is, the stained patches might be slightly larger than the expected lipid domains. Figure 2.7 shows the how the outline lipid layer on 90 nm C18SH Au NPs varies when the lipids are either POPS alone, 1:1 POPS/LPC, and LPC alone. With POPS alone, a complete coating of organics could be observed around the Au NP, while with LPC alone the organic layer was not observed, as it is
Figure 2.7. Preferential staining by OsO₄ for POPS on 90 nm C₁₈SH-coated Au NPs with (a) POPS alone [4, 90 nm], (b) 1:1 POPS/LPC [5, 90 nm], and (c) LPC alone [6, 90 nm]. Only POPS lipid domains are observed on Au NPs.
**Figure 2.8.** Stained TEM images of 20 nm PAH coated Au NPs with LPC and POPS [2, 20nm]. The difference in organic layer thickness could possibly indicate the presence bilayer or multilayer lipids. The organic layer is only visible when the Au NPs are not fully on the formvar coating of the TEM grid.
Figure 2.9. Cryo-TEM images of 20 and 90 nm Au NPs with an LPC coating ([3, 20 nm] and [3, 90 nm]).
not stained. A similar staining pattern was observed for both types of lipid-coated 50 and 90 nm Au NPs (Figure 2.10). With a nominal 1:1 POPS/LPC mixture, variations in staining patterns were observed. For both types of lipid-coated Au NPs of sizes 50 and 90 nm, patches of stained organics were observed, suggesting that domains of POPS are present on these NP surfaces as expected. The size of the patches is apparently, from the micrographs, a quarter to a half the circumference of the particles as they appear in the two dimensional images. Surprisingly, for mixed lipids on Au NPs of size 20 nm ([2, 20 nm] and [5, 20 nm]), a completely stained organic layer surrounding the NP was (faintly) observed. Mass spectrometry data supports the presence of LPC/POPS in ratios of 13:1 and 1:1 for hybrid and bilayer anchoring on 20 nm Au NPs respectively. This translates to patches with estimated coverage of 30° and 180° on these NPs, thus allowing us to rule out the patchy nature of lipids on 20 nm Au NPs, assuming the staining technique has sufficient resolution. This result then suggests that either the lipid layers are indeed random, not patchy, on these smaller particles or that only POPS binds significantly to the smaller NPs. However, the latter possibility is not likely from the mass spectrometry data; LPC is present at a much higher concentration than POPS for these Au NPs, thus ruling out that only POPS is present on the smaller Au NPs. This random distribution of POPS and LPC on smaller Au NPs (20 nm) and the patchy distribution on larger Au NPs (50 and 90 nm) suggests that surface curvature can influence the distribution and packing of lipids. A third possibility, of course, is that the TEM staining is insufficient to distinguish patches on 20 nm nanoparticles.

It has been clear for many years that the mixing of two lipids with different headgroups or acyl chains is nonrandom. In general, the demixing of two lipids can be understood from the contributions of the headgroups and the acyl chains. The headgroup of LPC is zwitterionic in character, with the negative charge at the phosphate separated by several angstroms from the
Figure 2.10. OsO₄-stained TEM images of PAH- and C₁₈SH-coated Au NPs of three different sizes with lipids, with the lipid being POPS alone, a mixture of POPS and LPC (1:1), and LPC alone. Scale bars = 15 nm. Only in the cases of 1:1 POPS/LPC are asymmetric coatings observed.
choline unit that carries the positive charge (\( \text{choline} \)).\(^{28}\) This is in contrast to POPS, which has a carboxylate group attached to the amine (\( \text{PO4}^{2-} \)). Evidence has shown that the PS–PS electrostatic repulsion can be overwhelmed by other interactions and that the mixing of PS and PC headgroup lipids is nonideal, that is, domains will form.\(^{26,27}\) The single cis double bond on one of the acyl chain in POPS also introduces a kink, imparting partial disorder properties which prevent close encounter with neighboring lipid.\(^{24}\) This causes POPS to pack poorly with LPC, which has a saturated acyl chain, further favoring its domain formation to minimize exposure of water to the hydrophobic regions.\(^{25,64}\) In addition, the tendency for lipids to phase-separate is increased with increasing curvature due to the reduction of lateral pressure and area density of lipids.\(^{20–22}\) When all these are taken into consideration, it is not surprising that mixed-lipid layers on Au NP surfaces will phase-separate to form domains of POPS and LPC rich regions, thus resulting in patchy NPs.

Parthasarathy et al. had previously shown a curvature of at least 0.8 ± 0.2 \( \mu \text{m}^{-1} \) can modulate the phase separation of lipids with the lipid domains preferred at lower curvatures.\(^{65}\) Here, we propose a possible mechanism for lipid adsorption onto Au NPs: due to the faceted nature of the surface of 50 and 90 nm diameter Au NPs, the curvature is extremely high at acute edges along facets and close to zero on the facets. POPS domains thus might only occupy positions on the facets, with the caveat that it is already not occupied by LPC. The formation of POPS domains is favored as they further reduce the surface energy by allowing tighter packing of lipids. The stronger electrostatic interaction of the PAH underlayer with POPS, compared to hydrogen bonding with LPC, might result in more POPS being included in the lipid bilayer on PAH-coated 50 and 90 nm Au NPs, which translated to a larger domain size as observed. The
facets on 20 nm diameter Au NPs are smaller. While POPS domains could still form on these facets, it might be energetically more unfavorable for the formation and stabilization of small POPS domains. In this sense, an even distribution of POPS and LPC would be preferred as compared to POPS domains, as the surface tension could be more evenly distributed between the conical-shaped LPC and the cylindrical-shaped POPS.

2.3.4. Functionalization of Lipid Coated 90 nm Au NPs with Smaller Au NPs via Protein Bridges

Lipid layer formation on Au NPs is not limited to POPS and LPC. Various surface modifications can be performed by incorporating functionalized lipids, either into the lipid layers of POPS or LPC alone or in the mixed lipid composition. This would ultimately improve and expand the functionality of Au NPs in areas such as targeting and imaging through control over the types of protein bound. As a proof of concept, we incorporated a biotinylated lipid, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl (biotin PE), at 25% (w/w) into the lipid composition. The lipids were then incubated with 90 nm Au NPs as per the above-mentioned protocol and purified. To determine the positioning of these biotin-functionalized lipids, anti-biotin antibodies conjugated to 12 nm Au NPs were incubated with the lipid-coated Au NPs and then purified by density gradient centrifugation. The spatial distribution of the small 12 nm Au NPs about the large 90 Au NPs was then visualized by TEM.

Our results demonstrate that biotin PE, through fingerprinting with 12 nm Au NP labels, is incorporated into the lipid bilayer and hybrid Au NPs (Figure 2.11). In addition, a range of distribution of 12 nm Au NPs for single and mixed lipids on Au NPs was seen. Figure 2.11a shows the change in 12 nm Au NP conjugation onto 90 nm PAH-coated Au NPs when the lipids were varied from POPS alone, mixed lipids, and LPC alone. In all cases, a 3:1 weight ratio was
Figure 2.11. (a) Schematic and TEM images of biotinylated 90 nm NPs with anti-biotinylated 12 nm NPs for lipid layers on PAH-coated Au NPs. Initial lipid ratios from top to bottom were POPS/PE (3:1), POPS/LPC/PE (1:2:1), and LPC/PE (3:1). (b) Schematic and TEM images of biotin/anti-biotin conjugation of 90 and 12 nm NPs for lipid layer on C18SH-functionalized Au NPs. Initial lipid ratios from top to bottom were POPS/PE (3:1), POPS/LPC/PE (1:2:1), and LPC/PE (3:1). All scale bars are 50 nm.
used where biotin PE makes up 25% (w/w). In the mixed lipid case, a ratio of 1:2:1 POPS/LPC/biotin PE was used. Likewise, the same was done for hybrid lipids on 90 nm Au NPs with POPS, mixed POPS/LPC, and LPC lipids (Figure 2.11b). TEM images demonstrate that at least two types of Au NP label distribution exists: individual 12 nm Au NPs and groups or patches of two or more Au NPs.

To quantify the distribution of 12 nm Au NP labels, we performed an angle analysis (Scheme 2.3). In this analysis, we can describe the distribution of the particles by either the average angle of labels or the distribution of label angles (Scheme 2.3). Simple mathematical modeling shows that the theoretical angle from the center of a 90 nm sphere to the edges of a 12 nm sphere is 13°. This allows us to identify individual particles as opposed to groups of particles from the angles. In addition, as a means to standardize measurements, 12 nm Au NPs separated by more than 1° were treated as individual particles or patches (more than 1 NP grouped together). The results of this analysis are shown in Table 2.2 for the data represented by Figure 2.11; histograms of the full data set are given in Figure 2.12. To characterize the distribution and extent of coverage, three parameters were calculated: (1) average number of patches per Au NP (avg. patches/NP) to show the distribution of biotin PE; (2) the fraction of patches ≥30° (no. of patches ≥30°/total) as a means to quantify the number of biotin PE domains, given that two particles together would provide an angle of 26°; and (3) the average angle covered on each NP (avg. angle per NP covered) to demonstrate the extent of coverage. Lipid composition on Au NPs influences the distribution of Au NP labels present. For example, for PAH-coated Au NPs, when the lipid composition was varied from POPS/biotin PE to POPS/LPC/biotin PE to LPC/biotin PE, the average number of patches per NP changed from 3.72 to 3.02 to 2.13. Of these patches, only 15.1% and 23.1% are larger than or equal to 30° (more than two particles
A cluster of small particles define the angle $\phi$, and the individual small particle defines the angle $\theta$. To distinguish between these two cases, small particles separated by more than $1^\circ$ are taken to be distinct patches. The average angle of all associated small particles can be calculated, or a histogram of measured angles per large particle can be calculated.
### Table 2.2. Distribution of Smaller Au NPs Bound to 90 nm Au NPs via Biotin/Anti-Biotin Interactions, for both PAH-Coated and C$_{18}$SH-Functionalized Au NPs$^a$

<table>
<thead>
<tr>
<th>system</th>
<th>PAH</th>
<th>Hybrid (C$_{18}$SH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POPS</td>
<td>POPS/LPC</td>
</tr>
<tr>
<td>total count 90 nm NP</td>
<td>107</td>
<td>160</td>
</tr>
<tr>
<td>total patches</td>
<td>398</td>
<td>483</td>
</tr>
<tr>
<td>avg. patches/NP</td>
<td>3.72</td>
<td>3.02</td>
</tr>
<tr>
<td>no. patches $\geq$ 30°/total</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>avg. angle per NP covered</td>
<td>75.2°</td>
<td>74.8°</td>
</tr>
</tbody>
</table>

$^a$Averages of 100 or more Au NPs were counted and the respective angles were measured.

“Patch” refers to a patch of small nanoparticles in this case. All lipid layers are synthesized with an initial biotin PE content of 25% (w/w).
Figure 2.12. Histograms of the counts of 12 nm Au NP label angles on 90 nm Au NPs with respect with the total angles measured. PAH coated Au NPs with a) POPS/ biotin PE, b) POPS/LPC/ biotin PE and c) LPC/ biotin PE. Hybrid Au NPs with d) POPS/biotin PE, e) POPS/LPC/ biotin PE and g) LPC/ biotin PE.
together) on Au NPs with POPS/biotin PE and POPS/LPC/ biotin PE, respectively, while for LPC/ biotin PE the ratio stands at 52.6%. Regardless of the type of patches formed, the mean angle occupied by labels per NP are similar: 75.2° for POPS/ biotin PE, 74.8° for POPS/LPC/ biotin PE, and 83.7° for LPC/ biotin PE. The distribution of labels on hybrid Au NPs are higher as compared to their PAH counterparts. The average number of patches per NP are higher at 4.76, 3.81, and 3.10 for POPS/ biotin PE, POPS/LPC/ biotin PE, and LPC/ biotin PE respectively. In addition, the mean angle occupied by labels per NP are also larger: 131.4° for POPS/ biotin PE, 107.3° for POPS/LPC/ biotin PE, and 98.7° for LPC/ biotin PE. However, the fraction of patches 30° or larger are similar for POPS/ biotin PE and LPC/ biotin PE at 33.9% and 39.6%, respectively, and lower for POPS/LPC/ biotin PE at 26.2%.

From this evidence, no clear preference of biotin PE for either hybrid or bilayer on Au NPs was observed. We can postulate that biotin PE had a preference for POPS as compared to LPC, on the basis of the data in Table 2.2. The larger number of patches with ≥30° on LPC-alone NPs could reflect an increased tendency of biotin PE to form separate domains within an LPC surface. It is clear that these data do once again suggest that lipid domain formation of biotin PE might occur on Au NP surfaces, thus highlighting the notion that the underlying lipid composition (POPS or LPC) can influence the distribution of the functionalized lipids. By carefully selecting the lipid types, compositions, and ratios, we believe that it is possible to fine-tune the distribution of functionalized lipids on Au NPs, such that the two extremes of ligand distribution (evenly dispersed versus Janus particles) can be achieved. Work along these lines is currently in progress.
2.4. Conclusion

Single and mixed lipid coating of Au NPs has been demonstrated, using two different means to secure the lipid onto the metal: hydrophobic interactions or electrostatics. Several lines of evidence, including UV−vis spectroscopy, ζ potential, and electron microscopy, support the formation of lipid layers on Au NPs. These lipid-coated Au NPs show remarkable stability to aggregation in buffers and at different pHs, which could certainly expand the possibilities for using Au NPs in biological applications for cellular imaging, targeting, or therapeutics. There is evidence for lipid domains on mixed-lipid-coated Au NPs larger than 50 nm, while evidence for a homogeneous mixed lipid layer was obtained for Au NPs of size 20 nm. Mass spectrometry of mixed-lipid-coated Au NPs reveals a large variation in lipid content that depends on initial surface coating of the gold and on gold core size. We have also shown that the lipid layer can be functionalized with biotin-conjugated lipids. The distribution of this biotin PE can be deduced through visualization after incubation with smaller Au NPs conjugated with anti-biotin antibodies. By careful selection of the types of lipids and ratios, the surface chemistry on Au NPs can be fine-tuned from an evenly dispersed configuration to that similar of Janus particles.

2.5. Acknowledgements

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CHAPTER 3

Wild-Type \( \alpha \)-Synuclein Binding and Orientation on Anionic Gold Nanoparticles*

3.1. Introduction

Parkinson’s disease (PD), a degenerative disorder of the central nervous system, affects \( \sim 2\% \) of individuals over the age of 60.\(^1\) This translates to about one million people in the United States in 2011, with 50 000 new cases diagnosed every year. PD is a movement disorder characterized by tremor, bradykinesia, muscular rigidity, and loss of coordination. Pathologically, the occurrence of PD is characterized by a loss of dopamine neurons in the substantia nigra pars compacta, coupled with the appearance of Lewy bodies, proteinaceous aggregates that develop inside nerve cells.\(^2\) Studies into these Lewy bodies have indentified \( \alpha \)-synuclein (\( \alpha \)-syn) as a major component of these inclusions,\(^3\) and many subsequent studies have shown that the overexpression of wild-type \( \alpha \)-syn and the production of wild-type and mutant \( \alpha \)-syn in mice and fly models lead to a loss of dopaminergic neurons, motor deficits, and neuronal inclusions, all of which are hallmarks of PD.\(^4,5\) A more recent in vitro study demonstrated that \( \alpha \)-syn can be transmitted from \( \alpha \)-syn-overexpressing (donor) cells to non-overexpressing (acceptor) cells, resulting in the formation of Lewy-like inclusions in acceptor cells.\(^6\) Although the cause of PD is still not known, these results show that the relationship between PD and \( \alpha \)-syn is deeply

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intertwined and imply that the severe disruption of protein homeostasis with the accumulation of misfolded protein in Lewy bodies can be the cause of neuronal death. As such, much effort has been expended in the study of α-syn.

α-Syn is a 140-amino-acid-long protein found in relatively high abundance in the brain (>1 μM in normal human brain and ~70 pM in cerebrospinal fluid). It is present in high concentration at presynaptic terminals and can be broadly divided into three domains: an amphiphilic N-terminus region (residues 1–60) that contains four imperfect 11-residue amino acid repeats with a conserved motif (KTKEGV), a hydrophobic, highly amyloidogenic domain (residues 61–95) with three additional KTKEGV repeats, and an acidic C terminus (residues 96–140). In aqueous buffers, α-syn is unstructured, but its shape can be easily modulated depending on the environment. When bound to vesicles composed of acidic lipids, α-syn adopts an α-helical structure. The curvature of the vesicles can influence the resultant α-helix: on small vesicles (20–25 nm), a horseshoe α-helix is maintained whereas on larger vesicles (~100 nm) an extended α-helix configuration is observed. At low pH, high temperature, or in the presence of metal ions, the premolten globule state is predominant. At high concentrations, α-syn tends to aggregate into a series of morphologically different soluble oligomers, which further fold into a β-sheet conformation that rapidly polymerizes into fibrils. It is these fibrils that form the basis of Lewy bodies. However, growing evidence suggests that it is not the fibrils but the oligomeric intermediates of α-syn that are the toxic species. Although the exact function (and hence native structure) of α-syn is still not known, several hypotheses for its function have been put forward: regulation of synaptic vesicle release and trafficking, modulation of membrane curvature, and physiological regulation of enzymes and proteins. The ability to control the various folding pathways of α-syn would thus enable us to relate structure to function more easily.
Interest in gold nanoparticles (Au NPs) has been building because of their potential use in therapeutics and biomedical applications. The combination of low toxicity, nanoscale size, and large scattering and absorption coefficients of Au NPs enable potential applications such as targeted contrast agents for photothermal therapy, drug delivery agents for cancer therapy, and transfection agents for gene therapy. In the context of α-syn, Au NPs are fascinating potential substrates (tunable from 12 to 100 nm) in the size range of the lipid vesicles that α-syn favors. However, fundamental questions about the interaction between Au NPs and the biological medium remain to be answered. Specifically, the Au NP-protein interaction can alter the way cells “perceive” Au NPs or proteins. For example, it has been shown that regardless of the initial surface functionalization on Au NPs, when introduced into cellular growth media loaded with bovine serum albumin (BSA), the resultant effective charge on Au NPs is the same as for BSA, signifying BSA adsorption. Dawson et al. have gone further to show that a plethora of proteins bind to NPs when introduced into human plasma, making up a two-layer protein corona: a hard inner corona of strongly associated and very slowly exchanging proteins and a soft outer corona of weakly bound proteins in rapid exchange with free proteins in solution. This resultant protein corona is likely how cells perceive NPs.

The formation of a protein corona on Au NPs is potentially a double-edged sword with regard to beneficial or detrimental effects on cells and will have to be carefully understood and perhaps regulated for future biomedical applications. First, low copy-number proteins, once trapped on the surface of Au NPs, may be rendered less bioavailable to the cell, thus throwing normal cellular pathways into disarray. Alternately, overexpressed proteins can be sequestered onto Au NPs and removed, making the cellular environment more “normal” in terms of the concentration of media components. Second, the adsorption of proteins onto Au NPs surfaces
can potentially alter the protein structure, which may result in severe consequences if such misfolded proteins trigger disease. Although many investigations assume that proteins (e.g., antibodies) maintain their tertiary structure when adsorbed onto NP surfaces, increasing emphasis has been placed on the changes that occur to protein structures in recent years.\textsuperscript{21,22} It can thus be inferred that the structural changes for natively unstructured proteins such as $\alpha$-syn might be more extreme when they are bound to Au NPs. These two points make the study of protein binding and structure on a nanoscale object an important and difficult task.

Thus far, work regarding $\alpha$-syn adsorption on NPs has been limited. $\alpha$-Syn has been used as a 1D assembly template to align Au NPs linearly or on Au NPs to use as a surface-enhanced Raman-scattering substrate.\textsuperscript{23,24} $\alpha$-Syn-conjugated quantum dots can accelerate $\alpha$-syn fibrillation in cells by acting as nucleating seeds.\textsuperscript{25} More work has been done on the amyloid $\beta$ peptide, a shorter aggregation-prone peptide also found in the brain. Protein fibrillation of amyloid $\beta$ can be controlled to a certain extent by influencing the lag phase, either by monomer or oligomer association to NPs.\textsuperscript{26} Amyloid fibrillation is also strongly dependent on the ratio of NP surface area to peptide concentration and the surface chemistry of the NPs.\textsuperscript{27,28} Molecular simulation has demonstrated that the shape and size of an NP can also influence amyloid fibril formation.\textsuperscript{29} These studies illustrate strong peptide binding to NPs and also provide indirect evidence that the specific adsorption of the peptide onto NPs occurred, making them more/less favorable to fibrillation and hence conformation change.

Here, we study the adsorption of wild-type $\alpha$-syn onto 20-and 90-nm-diameter Au NPs. Au NPs can be considered to be protein-sized substrates, thus allowing for a better understanding of the folding mechanism of $\alpha$-syn, or lipid vesicle mimics, allowing for an improved understanding of the shape control of $\alpha$-syn. As expected, $\alpha$-syn binds to Au NPs in multiple
layers via a hard and soft corona. The binding constants (overall and separate) were obtained using a combination of UV-vis spectroscopy, dynamic light scattering, and fluorescence quenching. We also demonstrate here that α-syn binds with higher affinity for 90 nm Au NPs than for 20 nm Au NPs that more closely match α-syn’s favored lipid vesicle substrate. In addition, we use a protease digestion foot printing method to deduce the structure of nanoparticle-bound α-syn.

3.2. Materials and Methods

3.2.1. Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium citrate tribasic dihydrate (Na₃Ct·2H₂O, ≥99%), hydroquinone (≥99%) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥99.5%) were obtained from Sigma-Aldrich and were used as received. Sequencing-grade trypsin (lot 082301) was obtained from GBioscience. Ultrapure deionized water (17.9 MΩ, Barnstead Nanopure II) was used for all solution preparations. A solution of HEPES buffer (20 mM, pH 7) was prepared in ultrapure deionized water. Trypsin solutions were made by reconstituting trypsin in (NH₄)₂CO₃ (25 mM) to a final concentration of 12.5 μg/mL (0.54 μM). Glassware was cleaned with aqua regia and rinsed thoroughly before use.

3.2.2. Synthesis of Gold Nanoparticles

Au NPs (diameter 20 nm) were synthesized by the boiling citrate method as previously described with some modifications.³⁰ Briefly, 2.5 mL of 0.01 M HAuCl₄·3H₂O was added to 97.5 mL of ultrapure water in a 200 mL Erlenmeyer flask and heated to boiling. An aqueous solution of sodium citrate (5% w/w, 2 mL) was then added, and the solution was allowed to boil for another 30 min. During this period, the color of the solution slowly changed to deep red.
Another portion of sodium citrate (1% w/w, 1 mL) was added, and the solution was allowed to boil for another 30 min. Au NPs produced by this method yielded a diameter of 17.5 ± 1.3 nm under transmission electron microscopy (TEM). The hydrodynamic diameter measured by dynamic light scattering was 31.2 ± 0.4 nm.

Au NPs (diameter 90 nm) were synthesized using 12 nm Au NPs as seeds and hydroquinone as the reducing agent. Seed Au NPs (diameter 12 nm) were synthesized using the boiling citrate method but with 3 mL of 1% sodium citrate. HAuCl₄·3H₂O solution (1% w/v, 2 mL) was centrifuged at 17,000 rcf for 1 h, after which the top 1 mL was obtained and added to 97.25 mL of ultrapure deionized water and 0.75 mL of the 12 nm NP solution. The solution was then stirred rapidly at room temperature, and 0.22 mL of 1% w/w sodium citrate was added, followed immediately by 1 mL of 0.03 M hydroquinone. The solution was allowed to stir overnight. Au NPs produced by this method yielded a diameter of 89 ± 13 nm under TEM. The hydrodynamic diameter measured using dynamic light scattering was 90.8 ± 0.3 nm.

3.2.3. Production of α-Synuclein

α-Syn protein was expressed and purified as previously described. Briefly, the gene encoding wild-type human α-syn (SNCA) was cloned into the pET28 vector (Novagen). Protein expression was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 37 °C, followed by alkaline lysis, boiling, and precipitation with 60% ammonium sulfate. Precipitates were resolubilized and purified by hydrophobic interaction chromatography on a HiPrep 16/10 butyl fast flow Sepharose column (GEBiosciences) and eluted with decreasing salt. α-Syn-containing fractions were pooled and concentrated, subjected to size-exclusion chromatography on a Superdex 75 10/300 GL column (GEBiosciences), and lyophilized for storage at −80 °C.
The concentrations of α-syn solutions were determined by measuring the UV absorbance at 280 nm using an extinction coefficient of 5200 M⁻¹cm⁻¹.33

3.2.4. Incubation of α-Synuclein with Gold Nanoparticles

Prior to each experiment, an aliquot of α-syn was reconstituted in HEPES buffer at a 1 mg/mL (70 μM) concentration. For dynamic light scattering (DLS) and UV–vis measurements, purified Au NPs (for 20 nm, 1.2 nM in particle concentration for DLS and 0.63 nM for UV–vis; for 90 nm, 10.5 pM for both DLS and UV-vis) were added and incubated with varying concentrations of α-syn (0–7 μM) at 4 °C for at least 12 h to ensure equilibrium. For fluorescence quenching measurements, varying concentrations of Au NPs (for 20 nm Au NPs, from 0 to 0.16 nM; for 90 nm Au NPs, from 0 to 5 pM) were incubated with α-syn (7 μM). All measurements were made at 25 °C before returning the samples to 4 °C for storage. The hard-corona bound α-syn on Au NPs was purified from excess free α-syn and soft corona-bound α-syn by centrifugation at least three times and dispersed in HEPES buffer.

3.2.5. Amino Acid Analysis

α-Syn-coated Au NPs were purified a minimum of three times via centrifugation. An aliquot of a protein–nanoparticle complex of known concentration was digested with either 1 M KCN or aqua regia. The sample was then dried down and resuspended in constant-boiling HCl (6 N) in a sealed ampule at 110°C for 12–24 h. The sample was again dried down in a speed vacuum prior to derivatization, and amino acids were quantified by gas chromatography–mass spectrometry (GC-MS). Because the amino acid sequence of α-syn is known, the α-syn per Au NP can be deduced.
3.2.6. Trypsin Digestion of α-Synuclein on Au NPs

α-Syn-coated Au NPs were purified a minimum of three times via centrifugation. Fixed volumes of trypsin solution were added to known number of α-syn coated Au NPs such that the ratio of trypsin/α-syn ranged from 1:5 to 1:160. The samples were microwave digested for 15 min at 55 °C (70 W), following which separation was achieved using Amicon filters (MWCO = 30 000 Da) with centrifugation at 14,000 rcf for 5 min. The supernatant was collected, dried down, and analyzed using nanoliquid chromatography mass spectrometry/mass spectrometry (LC MS/MS). The Au pellet was further purified twice before the digestion of the Au NPs with 1 M KCN, lyophilized to dryness, and analyzed. The mass spectrometry data was processed using Waters Protein Lyns Global Server 2.2.5, Mascot (Matrix Sciences) and blasted against the NCBI-NR protein database for wild-type human α-syn (gi|4507109).

3.2.7. Instrumentation

A microcentrifuge (Eppendorf model 5418, Fisher-Thermo Electron) was used in various steps of synthesis and purification as detailed above. Standard absorption spectra were taken on a Cary 500 Scan UV–vis–NIR spectrophotometer at a scan rate of 30 nm/s. More accurate scans were performed at a 1 nm/s scan rate from wavelengths 450 to 550 nm for 20 nm Au NPs and from 530 to 595 nm for 90 nm Au NPs. Zeta potential and dynamic light scattering measurements were performed on a Brookhaven Zeta PALS instrument. Fluorescence quenching data were obtained on a Jobin Yvon FluoroMax-3 fluorometer. The excitation wavelength was set at 270 nm, and the emission was scanned from 275 to 400 nm at a scan rate of 1 nm/s and an integration time of 0.1 s. All spectra were corrected with HEPES buffer as the baseline. Areas under the emission peak were used to quantify the fluorescence. All measurements were made in
triplicate. Amino acid analysis was performed on an Agilent 6890 gas chromatograph (with an Agilent 7683B autosampler) coupled to an Agilent 5973 mass-selective detector. The column used was a 30 m DB5 column, and the helium gas was set at a constant flow rate of 3 mL/min. The mass spectrometer was operated in positive electron impact mode (EI) at a 69.9 eV ionization energy in the m/z 50−800 scan range. The spectra of all chromatogram peaks were evaluated using AMDIS (NIST, Gaithersburg, MD, USA). Protein digestion was performed on a CEM Discover microwave digestor (Mathews, NC). The mass spectrometer used for peptide analysis was a Waters quadrupole time-of-flight mass spectrometer (QToF) connected to a Waters Nano Acquity UPLC. A Waters AtlantisC-18 (0.03 mm particle, 0.075 mm × 150 mm) column was used, with the flow rate set to 250 nL/min. Peptides were eluted using a linear gradient of water/acetonitrile containing 0.1% formic acid 0−60% B in 60 min. The mass spectrometer was set for data-dependent acquisition; ms/ms was performed on the most abundant four peaks at any given time.

3.3. Results and Discussion

3.3.1. General Binding of α-Syn to Au NPs

The hydrodynamic diameter of α-syn is 5.3 ± 1.0 nm, and the two sizes of Au NPs used as its substrates were 20 nm (with a surface coating of citrate ions) and 90 nm Au NPs (made in the presence of sodium citrate and hydroquinone). One method to monitor protein adsorption was dynamic light scattering (DLS). The incubation of citrate-capped 20 nm Au NPs (original DLS diameter 31.2 ± 0.4 nm) with excess α-syn showed a Gaussian distribution of hydrodynamic sizes with a huge increase in mean diameter to 52.4 ± 1.6 nm before centrifugation (Figure 3.1a). The distribution shifted to a smaller mean of 46.4 ± 0.5nm after centrifugation, which led to a decrease in diameter of about 6.0 nm. The incubation of 90 nm Au
Figure 3.1. Dynamic light scattering (DLS) plots of α-syn-coated Au NPs with initial diameters of 20 and 90 nm under transmission electron microscopy. The hydrodynamic diameter as measured by DLS of the 20 nm Au NPs was 31.2 ± 0.4 nm, and that for 90 nm Au NPs was 90.8 ± 0.3 nm. All distributions were fitted with Gaussian curves. (a) DLS plots of α-syn-coated 20 nm Au NPs before (black) and after (red) centrifugation. (b) DLS plots of α-syn-coated 90 nm Au NPs before (black) and after (red) centrifugation. (c) DLS plots of α-syn-coated Au NPs (black) after a series of dilutions with HEPES buffer (red, green, and blue) at 1.5-, 2.0-, and 2.5-fold dilution, respectively. The mean size decreased as more HEPES buffer was added.
NPs (original DLS diameter 90.8 ± 0.3 nm) with excess α-syn demonstrated a similar increase in hydrodynamic diameter to 105.9 ± 1.0 nm before centrifugation (Figure 3.1b). With centrifugation, a decrease of 5 nm to 100.9 ± 0.6 nm was observed. This suggests that α-syn on Au NPs adsorbs in multiple layers: a strongly bound inner (hard) layer (15 nm thick on 20 nm Au NPs and 10 nm thick on 90 nm Au NPs) and a relatively weakly adsorbed outer (soft) layer (6 nm thick on 20 nm Au NPs and 5 nm thick on 90 nm Au NPs). The outer “soft” α-syn corona is weakly bound and labile and strongly depends on the concentration of free α-syn: when 20 nm Au NPs incubated with excess α-syn were slowly diluted with HEPES buffer, these α-syn-coated Au NPs, having a mean diameter of 56.1 ± 1.0 nm before dilution, were reduced to a minimum of 48.1 ± 0.7 nm after three dilution steps at 0.5× dilution each step (Figure 3.1c). By titrating α-syn into Au NP solutions and monitoring the adsorption of α-syn to 20 and 90 nm Au NPs using DLS and, independently, UV–vis spectroscopy to monitor plasmon bandshifts of the Au NPs, we can determine the overall binding constant of α-syn to Au NPs as well as estimate the total number of α-syn bound per Au NP.

Increasing amounts of α-syn increased the initial hydrodynamic diameter of the 20 nm Au NPs from 31.2 ± 0.4 nm to a maximum of 55.5 ± 0.3 nm (Figure 3.2a). Further addition of α-syn did not significantly increase the overall particle size, signifying the total saturation of the Au NP surface by α-syn. No breaks in the data were observed as a function of protein concentration, suggesting that the formation of the hard and soft coronas of α-syn onto Au NPs is not a simple two-step process. The point of saturation obtained from the plateau of the DLS plot was about 460 α-syn adsorbed per Au NP total in both the hard and soft coronas. An overall binding constant was estimated from the DLS data by fitting it to a Langmuir adsorption isotherm model.
Figure 3.2. Titration of α-syn into Au NP solutions. (a) Hydrodynamic diameter changes of 20 nm Au NPs with an increasing α-syn/Au NP ratio. α-Syn (0–7 μM) was added to 1.2 nM of a 20 nm Au NP solution. (b) UV–vis spectral changes with increasing amounts of α-syn (0–7 μM) added to a 0.63 nM 20 nm Au NP solution. (Inset) Change in absorption peak maxima with increasing α-syn/NP ratio. (c) Hydrodynamic diameter changes of 90 nm Au NPs with an increasing α-syn/Au NP ratio. α-Syn (0–7 μM) was added to 10.5 pmoles of 90 nm Au NP solution. (d) UV–vis spectral changes with increasing α-syn (0–7 μM) added to a 10.5 pM 90 nm Au NP solution. (Inset) Change in absorption peak maxima with increasing α-syn/NP ratio. The error bars in the DLS data represent one standard deviation from the mean of at least three independent experiments.
where $\Delta D$ and $\Delta D_{\text{max}}$ are the change and maximum change in NP diameter, $K_a$ is the association constant, and $[\alpha\text{-syn}]$ is the concentration of $\alpha$-syn for each DLS measurement point. If we assume that the increase in hydrodynamic diameter is attributed only to the adsorption of $\alpha$-syn alone, an overall binding constant of $(2.0 \pm 0.4) \times 10^7 \text{ M}^{-1}$ was obtained. Here, because the Langmuir model assumes a homogeneous single layer $\alpha$-syn adsorption onto Au NPs, no distinction between the hard and soft coronas is made.

The plasmon band maxima in the UV-vis spectra of 20 nm Au NPs shifted gradually from 522 nm to a maximum of 527 nm with the gradual addition of $\alpha$-syn (Figure 3.2b). The shift in the peak maximum of the plasmon band of Au NPs is due to the local refractive index change by the adsorption of $\alpha$-syn.$^{35}$ Assuming that the shift was due to only the $\alpha$-syn-induced refractive index change and each $\alpha$-syn binds to only one type of surface site on Au NPs, this shift was fitted into a Langmuir isotherm

$$\frac{\Delta \lambda}{\Delta \lambda_{\text{max}}} = \frac{K_a[\alpha\text{-syn}]}{1 + K_a[\alpha\text{-syn}]}$$

where shifts in the plasmon peak position are used instead of the DLS data from eq 1. From this spectroscopic data, a $K_a$ of $(1.2 \pm 0.3) \times 10^7 \text{ M}^{-1}$ was obtained. When the plasmon band maxima were plotted with respect to $\alpha$-syn/Au NP ratio, a saturation ratio of 380 $\alpha$-syn/Au NP was obtained, similar to that obtained using DLS.

A similar trend was observed on 90 nm Au NPs (Figure 3.2c,d). The titration of $\alpha$-syn to 10.5 pM 90 nm Au NPs increases the hydrodynamic diameter from 90.8 ± 0.3 nm in the absence of $\alpha$-syn to a maximum of about 110.4 ± 1.0 nm. The binding constant was estimated to be $(1.8 \pm 0.2) \times 10^8 \text{ M}^{-1}$, with 40 000 $\alpha$-syn/NP at saturation. In UV−vis spectroscopy, a gradual plasmon band shift from an initial 561.3 nm to a final 564.5 nm was observed, which correlated to a
binding constant of \((2.0 \pm 0.2) \times 10^8\) M\(^{-1}\). A saturation point of 60 000 \(\alpha\)-syn/Au NP was obtained from the peak maxima change. Previous work has shown that \(\alpha\)-syn can bind to silica and polymeric NPs; however, most examples use antibodies to capture and sequester \(\alpha\)-syn.\(^{36,37}\) \(\alpha\)-Syn has also been mutated with a cysteine residue to exploit the strong gold-sulfur bond for its covalent attachment to Au NPs.\(^{23}\) Our results showed that the adsorption of WT \(\alpha\)-syn to citrate-capped 20 nm Au NPs and citrate-/hydroquinone-coated 90 nm Au NPs occurred in a multilayered fashion: a strongly bound hard \(\alpha\)-syn corona followed by a weaker bound soft corona. Two independent methods, UV–vis spectrometry and DLS measurements, yielded very similar overall binding constants for both 20 and 90 nm Au NPs, respectively (Table 3.1). Wavelength shifts from UV–vis spectra have been shown to be a reliable indicator of physical and chemical changes at the NP surface.\(^{35,38}\) Such shifts can be converted to binding constants with the use of the Langmuir isotherm and have been widely used to quantify protein binding on Au NPs: bovine serum albumin absorption affinity onto 15 nm Au NPs was determined to be between 1 × 10\(^6\) and 5 × 10\(^7\) M\(^{-1}\).\(^{39}\) Similarly, DLS measurements have also been used to quantify the extent of binding.\(^{40,41}\) It should be noted that the Langmuir model is the simplest possible model for obtaining binding constants for the adsorption of molecules to a surface and does not take into account multilayered adsorption. However, the more complex adsorption isotherms that we have examined introduce more fitting parameters and therefore more uncertainty in comparing the overall binding constants without any improvement in the degree of fit. On the basis of previous work for DNA-quantum dot adsorption using various binding constant models, the Langmuir model should still provide a good estimate of the binding strength of \(\alpha\)-syn on Au NPs within an order of magnitude of the “true” value.\(^{42}\)
Table 3.1. Binding of α-Syn onto Au NPs or Lipid Vesicles

Summary of Binding of α-Syn on Au NPs (20 mM HEPES)

<table>
<thead>
<tr>
<th>Au NP</th>
<th>Total Corona</th>
<th>Hard Corona</th>
<th>Soft Corona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>binding constant (M⁻¹)</td>
<td>α-syn/Au NP binding constant (M⁻¹)</td>
<td>Fluorescence (Hill)</td>
</tr>
<tr>
<td></td>
<td>UV-vis</td>
<td>DLS</td>
<td>AAA</td>
</tr>
<tr>
<td>20 nm</td>
<td>(1.2 ± 0.3) x 10⁷</td>
<td>(2.0 ± 0.4) x 10⁷</td>
<td>(2.0 ± 0.2) x 10⁸</td>
</tr>
<tr>
<td></td>
<td>380</td>
<td>460</td>
<td>60 000</td>
</tr>
<tr>
<td></td>
<td>(2.9 ± 1.1) x 10⁹</td>
<td>(2.3 ± 1.4) x 10⁹</td>
<td>(9.5 ± 0.8) x 10¹⁰</td>
</tr>
<tr>
<td></td>
<td>360 ± 70</td>
<td>n = 0.79 ± 0.04</td>
<td>5300 ± 700</td>
</tr>
</tbody>
</table>

Summary of Binding of α-Syn on Lipid Vesicles

<table>
<thead>
<tr>
<th>lipid vesicle size (nm)</th>
<th>lipid type</th>
<th>binding constant (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30–50⁹</td>
<td>DPPC</td>
<td>2.6 x 10⁷</td>
</tr>
<tr>
<td>46³</td>
<td>POPC/POPG (1:1)</td>
<td>3.5 x 10⁷</td>
</tr>
<tr>
<td>93³</td>
<td>POPC/POPS (1:1)</td>
<td>1.8 x 10⁵</td>
</tr>
<tr>
<td>162³</td>
<td>POPC/POPS (1:1)</td>
<td>1.0 x 10⁴</td>
</tr>
</tbody>
</table>

¹1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS). ²Reference 48. ³Reference 49.
The multilayered binding of α-syn to Au NPs is in contrast to other protein binding reported in the literature. Although multilayered adsorption is observed when a plethora of proteins is present (such as in cell culture media), most work on single protein adsorption on nanoparticles demonstrated only the presence of a monolayer. For example, human serum albumin (HSA) was shown to exist as a monolayer on FePt nanoparticles. However, it should be pointed out that unlike HSA, which has a well-defined secondary and tertiary structure, native α-syn is unstructured and is known to aggregate. Small changes in α-syn structure, a result of electrostatic interactions between the Au NP and α-syn in the hard corona, can potentially influence its protein-protein interaction, thus forming a soft corona.

3.3.2. Quantification of the Hard α-Syn Corona on Au NPs

The DLS results suggested that two types of α-syn adsorption exist: a hard corona that binds directly and tightly to the nanoparticle surface and a soft corona that is loosely bound and is likely in rapid equilibrium with free protein. This implies that there should be two average equilibrium binding constants for each corresponding hard and soft layer. The hard α-syn corona on Au NPs can be separated from the soft corona via centrifugation, and the hard corona remains tightly bound on the Au NP surface even after multiple centrifugation cycles. This fact can be exploited and used to purify Au NPs coated with a hard corona of α-syn. This α-syn in the hard corona was collected after selective Au dissolution and quantified more precisely using mass spectrometry, affording a value of 360 ± 70 α-syn per 20 nm Au NP and 5300 ± 700 α-syn per 90 nm Au NP in the hard corona, within the combined total of α-syn estimated from DLS and UV−vis spectroscopy.

In comparison to the DLS data, the difference in the α-syn corona for 20 and 90 nm Au NPs is further highlighted (Table 3.1). The hard corona on 20 nm Au NPs is thicker (15 nm) than
that on the 90 nm Au NPs (10 nm). Simple calculations, assuming α-syn as spheres with a 5.3 nm diameter, suggest an estimate of about 60 and 1200 α-syn molecules for a monolayer of coverage on 20 and 90 nm Au NPs, respectively. The 6- and 4.4-fold excesses of α-syn in the hard corona on 20 and 90 nm Au NPs, respectively, possibly imply either multilayered adsorption of α-syn in the hard corona or a significant rearrangement of α-syn structure/orientation such that tighter packing is ensured. This result is similar to that of β2-microglobulin, a fibrillation-prone protein, on polymeric nanoparticles that shows that multilayers of protein can form on nanoparticle surfaces.44

DLS measurements suggest that the soft corona is similar in thickness (5 to 6 nm) for 20 and 90 nm Au NPs. Interestingly, a comparison of total and hard coronas suggests that about 30–100 α-syn are in the soft corona for 20 nm Au NPs and about 35,000–55,000 α-syn for 90 nm Au NPs, translating to about 14 and 85% of the total α-syn in the soft corona, respectively. An approximate of 90 and 1280 α-syn was estimated for the soft corona on 20 and 90 nm Au NPs for a thickness of 5 to 6 nm. This value is similar to that found in the soft corona for 20 nm Au NPs; however, for 90 nm Au NPs, a 35-fold excess of α-syn was found compared to the calculated value. The difference between found and expected α-syn in the soft corona of 20 and 90 nm Au NPs suggests that the Au NP size can influence the dynamics of protein–protein interaction even when not directly absorbed onto the Au NP surface. Such effects are important and should be considered when studying the impact of Au NPs on protein expression in cells.

3.3.3. Fluorescence Quenching Study of the Hard α-Syn Corona on Au NPs
To quantify the binding affinity of the hard corona for Au NPs, a two-step fluorescence quenching experiment was performed (Scheme 3.1). In the first step, varying concentrations of
Scheme 3.1. Cartoon of Au NPs with Hard (Blue) and Soft (Pink) Coronas and the Techniques Used to Deduce α-Syn Binding Constants and Structure/Orientation

Note that the horseshoe structure of α-syn shown here is not representative of its actual conformation on Au NPs.
Au NPs were titrated against 7 μM α-syn such that the final concentration of the Au NPs ranged from 0 to 0.16 nM for 20 nm Au NPs and 0 to 5 pM for 90 nm Au NPs. As expected, increasing concentrations of Au NPs resulted in decreasing protein fluorescence intensities (Figure 3.3a). Plotting $F_0/F$, the ratio of initial protein fluorescence to protein fluorescence at a given NP concentration, against $[\text{Au NP}]$ yielded a curved plot that can be attributed to the strong absorption of Au NPs at the excitation and emission wavelengths in addition to quenching due to protein–nanoparticle binding, greatly reducing the fluorescence of unbound α-syn even when α-syn is present in very large excess. To account for this inner-filter effect by the Au NPs, the α-syn-coated Au NPs used in this first titration were collected, purified, and used in the second titration set against the same amount of α-syn at 7 μM (second step). Because this titration set consist of Au NPs precoated with a hard corona of α-syn, the fluorescence quenching obtained was assumed to be due to a combination of the Au NPs’ light absorption, again, and the soft corona (Figure 3.3a). The difference between the first and second fluorescence plots is then due solely to the hard corona of α-syn on Au NPs. The calculation of the hard corona binding constant was achieved by using the second plot to derive a correction factor ($\theta$) for each Au NP concentration

$$\theta = \frac{F}{F_0}$$  \hspace{1cm} (3)

where $F$ is the protein fluorescence intensity at the respective Au NP concentration and $F_0$ is the initial protein fluorescence intensity. The $\theta$ factor ranges from 0.76 to 1 and from 0.45 to 1 for the range of 20 and 90 nm Au NP concentrations used, respectively, reflecting the larger self-absorption of fluorescence (and hence smaller $\theta$ value) by 90 nm Au NP due to its extremely large extinction coefficient. The influence of the soft corona and Au absorption can then be factored and removed from the first fluorescence plot by dividing the fluorescence intensities by
**Figure 3.3.** (a) Fluorescence quenching of 7 μM α-syn by 20 nm Au NPs (black) and α-syn-coated 20 nm Au NPs (red). (b) Fluorescence quenching plot of 20 nm Au NPs before (black) and after (red) correction (i.e., the removal of Au absorption and soft corona fluorescence quenching). (c) Fluorescence quenching of 7 μM α-syn by 90 nm Au NPs (black) and α-syn-coated 90 nm Au NPs (red). (d) Fluorescence quenching plot of 90 nm Au NPs before (black) and after (red) correction. The fluorescence intensity was taken as the area under the curve of the protein’s emission spectrum from 275 to 400 nm upon excitation at 270 nm.
their respective $\theta$ such that a “corrected” plot of fluorescence quenching due to the formation of a hard corona remains (Figure 3.3b). This plot is in turn used to estimate the binding constant for the hard corona. Assuming static quenching, the fluorescence quenching plot of $\alpha$-syn hard corona can be fitted into a static quenching equation

$$\frac{F_0}{F_c} = 1 + K_s[Au \text{ NP}]$$

(4)

where $F_c$ is the corrected fluorescence intensity at the respective Au NP concentration. This yielded a straight line plot with $K_s$ values of $(2.9 \pm 1.1) \times 10^9 \text{ M}^{-1}$ and $(9.5 \pm 0.8) \times 10^{10} \text{ M}^{-1}$ for 20 and 90 nm Au NPs, respectively.

Alternatively, the Hill equation is frequently used as a measure of the binding cooperativity of ligands onto the same macromolecule and is given as

$$\frac{(F_0-F_c)}{(F_0-F_\infty)} = \frac{[Au \text{ NP}]^n}{K_D^n + [Au \text{ NP}]^n}$$

(5)

where $F_c$ is the corrected fluorescence intensity of the protein, $F_0$ is the initial protein fluorescence intensity, $F_\infty$ is the fluorescence intensity at saturation, $n$ is the Hill coefficient, and $K_D$ is the equilibrium constant. The association binding constant $K_a$ is the reciprocal of $K_D$. The Hill coefficient, $n$, can be regarded as an “interaction” coefficient that reflects the extent of cooperativity among binding sites, with $n > 1$ signifying positive cooperativity, $n < 1$ signifying negative cooperativity, and $n = 1$ signifying noncooperativity. Fitting the corrected fluorescence quenching data into a Hill plot revealed $K_a = (2.3 \pm 1.4) \times 10^9 \text{ M}^{-1}$ and $n = 0.79 \pm 0.04$ for 20 nm Au NPs and $K_a = (3.5 \pm 0.5) \times 10^{10} \text{ M}^{-1}$ and $n = 0.5 \pm 0.1$ for 90 nm Au NPs, signifying that $\alpha$-syn binds strongly to both 20 and 90 nm Au NPs and this binding affinity decreases as further proteins adsorb onto the surface.

Interestingly, our results show that $\alpha$-syn in the hard corona adsorbs more strongly onto Au NPs as the NP size increases. A similar trend was observed with the overall binding constant:
an increase from \((1.2 - 2.3) \times 10^7 \text{ M}^{-1}\) for 20 nm Au NPs to \((1.8 - 2.0) \times 10^8 \text{ M}^{-1}\) for 90 nm Au NPs. These numbers are large compared to \(\alpha\)-syn binding to small lipid vesicles, composed of either a combination of phosphocholine and phosphoglycerol or phosphocholine alone at 25 or 37 °C, with \(K_a\) values of \(2.6 \times 10^7\) and \(3.5 \times 10^7 \text{ M}^{-1}\), respectively (ionic strength, \(I = 120 \text{ mM}\)).\(^{46}\)

Giant unilamellar vesicles of diameter 93 and 162 nm have \(K_a\) values of \(1 \times 10^4\) and \(7.4 \times 10^3 \text{ M}^{-1}\), respectively, for \(\alpha\)-syn \((I = 160 \text{ mM})\).\(^{47}\) In our experiments, the salt strength was maintained at 20 mM. Although it is known that the ionic strength can influence the binding affinity of \(\alpha\)-syn to nanoscale objects,\(^{48}\) we believe that ionic strength changes cannot solely account for differences in \(K_a\) values of about 5 orders of magnitude for 90 nm particles. This highlights that other factors are involved in the enhanced absorption of \(\alpha\)-syn to 90nm Au NPs.

Quantification of the binding constant of the soft protein corona to NPs, is, however, nontrivial. Because of the highly dynamic nature of the soft corona, the soft corona cannot be explicitly separated from the unbound free \(\alpha\)-syn. In addition, fluorescence inner-filter effects by Au NPs cannot be separated from the fluorescence quenching effect of the weakly bound \(\alpha\)-syn in the soft corona. The nonlinear effect of fluorescence quenching by Au NPs also falls off exponentially with respect to distance (unless one is in the regime where surface-enhanced fluorescence can take place, approximately 10–20 nm away from the metal surface). All of these factors make the determination of the soft corona binding using UV–vis, DLS, and fluorescence measurements complicated. However, an estimate of the soft corona binding constants can be obtained from the comparison of the combined adsorption constants (Langmuir isotherm) and hard corona adsorption constants (static quenching model). Here, the combined adsorption constant is taken to be a product of the hard and soft adsorption constants \((K_{\text{overall}} = K_{\text{hard}} \times K_{\text{soft}})\). This gives \(K_{\text{soft}}\) values of \((7.9 \pm 5.0) \times 10^{-3} \text{ M}^{-1}\) for 20 nm Au NPs and \((2.1 \pm 0.4) \times 10^{-3} \text{ M}^{-1}\) for
90 nm Au NPs (Table 3.1). These numbers suggest that the binding of the soft corona is thermodynamically unfavorable by \(\sim 2\) kJ/mol and therefore that soft corona binding is kinetically driven, highly dynamic, and in constant exchange with “free” \(\alpha\)-syn in solution.\textsuperscript{49}

3.3.4. Structure of Bound \(\alpha\)-Syn in the Hard Corona

Currently, numerous methods for studying the protein conformation on curved Au NP surfaces are available. Circular dichroism (CD) can be used to detect proteins on Au NPs, although the background from free protein can render the data difficult to interpret; therefore, the best use of CD seems to be if the free protein has no CD signal but the bound protein has a strong one.\textsuperscript{50} We performed double-cuvette experiments where two cuvettes are stacked together and the samples are added to either of the cuvettes (Figure 3.4a). This setup allows us to isolate the structural change in \(\alpha\)-syn when it is adsorbed onto Au NPs as opposed to the light absorption of Au NPs. However, apparent CD changes in \(\alpha\)-syn signals in the presence of Au NPs were found to be trivial (Figure 3.4b). The high absorbance of metallic NPs can be overcome with the use of more exact but less accessible techniques such as synchrotron radiation circular dichroism.\textsuperscript{51} More conventional spectroscopy experiments have provided some insight into the protein structure on colloidal nanoparticle surfaces; for example, infrared spectra of the amide I band have been used to probe the secondary structure of hemoglobin on Au NPs.\textsuperscript{52} NMR had been used to study the ubiquitin interaction on 12 nm Au NPs.\textsuperscript{53} More recently, MALDI-MS was used to study the orientation of proteins on silica NPs.\textsuperscript{22} Here, we introduce a footprinting method to study the protein orientation on NPs that involves the use of an enzymatic digestion step to probe solvent-accessible portions of the bound protein. While our studies were in progress, related work by Shrivastava et al. concurs that enzymatic digestion followed by a mass
Figure 3.4. (a) Setup for the circular dichroism (CD) experiments. Two cuvettes (1 mm) are stacked together, and the samples are placed in cuvette A or B. (b) CD spectra of α-syn alone (black, 10 μM), α-syn and Au NPs (20 nm, 5.6 nM) in separate cuvettes (red), and α-syn absorbed onto Au NPs (blue). MES buffer (10 mM, pH 6.5) was used, and the average of 10 scans was taken (1 nm bandwidth, 12 nm/min). On the basis of the 1:360 Au NP/α-syn binding ratio, 20% of α-syn would be bound.
spectrometry analysis of nanoparticle-bound protein can give insight into protein orientation on nanoparticle surfaces. Here we show that this method is especially useful for strongly colored NPs such as Au NPs.

To deduce the structure of α-syn on Au NPs, we performed in situ protein digestion of α-syn on Au NPs. For the case of α-syn, although the lipid-bound α-helical horseshoe structure of α-syn was deduced using NMR, its native and β-sheet structures are relatively unknown. We hypothesized that proteases would attack a protein differently when on Au NP surfaces as compared to its unbound free form in solution. This difference in digestion can then be utilized to study the structure of the protein on Au NPs as a footprinting assay and thus deduce its orientation. In our study, we used trypsin to cleave α-syn at its lysine positions (there are no arginine residues, another site of trypsin attack, in α-syn; Figure 3.5a). A total of 15 lysines are on α-syn at positions 6, 10, 12, 21, 23, 32, 34, 43, 45, 58, 60, 80, 96, 97, and 101. The peptide fragments that were liberated in solution were collected separately from those bound to the Au NPs, and both samples were quantified using LCMS. When compared against the free α-syn digestion with trypsin, these differences would then yield (1) the section of α-syn that was bound to the Au NP surface, (2) lysine positions that were not cleaved in the same time frame, signifying a more “protected” region, and (3) the ratio of peptide fragments that reveal the extent of protection. By varying the trypsin/α-syn ratio, a clearer picture of the extent of protection can be obtained, which can be reconstructed to deduce the structure and orientation of α-syn on Au NPs in the hard corona. However, because of the random nature of protease digestion, multiple experiments must be done to ensure reproducibility (Figure 3.6). Nevertheless, in all cases, although the absolute number of peptides detected varied, the ratio and peptide sequences were very similar under each experimental condition. Figure 3.7a shows the digestion patterns of free
Figure 3.5. \( \alpha \)-Synuclein amino acid sequence and the preferred digestion sites of trypsin on (a) free \( \alpha \)-synuclein, (b) \( \alpha \)-synuclein adsorbed on 20 nm Au NPs, and (c) \( \alpha \)-synuclein adsorbed on 90 nm Au NPs. The trypsin/\( \alpha \)-synuclein ratio shown here is 1:160. Lysine positions are highlighted in red, and sites susceptible to trypsin digestion are shown by the arrows. The frequency of trypsin attack is shown as a color scale, with black being the easiest for trypsin to attack and light gray being the least digested.
Figure 3.6. Replicates of trypsin digestion of α-syn bound on 20 nm Au NPs at trypsin:α-syn ratios of (a) 1:160, (b) 1:100 and (c) 1:5. This demonstrates the reproducibility of trypsin digestion of α-syn on Au NPs.
**Figure 3.7.** (a) Digestion pattern of free α-syn with trypsin at various trypsin/α-syn ratios. Digestion pattern of α-syn adsorbed on 20 nm Au NPs at trypsin/α-syn ratios of (b) 1:160, (c) 1:100, and (d) 1:5. The peptide fragments in the supernatant are separated from those on the Au NP surface and are shown separately. The horizontal axis denotes the amino acids in α-syn from the N to C terminus. The vertical axis denotes the different types of peptide fragments obtained, which is further separated into those in the supernatant and on the Au NP surface. The color scale (normalized) denotes the number of peptides found. The scale on the z axis is the same for all plots.
α-syn at trypsin/α-syn ratios of 1:160, 1:100, and 1:5. The number of peptides was normalized with respect to the highest peptide count for ease of comparison. The peptide fragments are arranged according to their amino acid positions. These results show that increasing the ratio of trypsin/α-syn did not change the digestion pattern of free α-syn significantly, suggesting that under our experimental conditions the digestion of free α-syn was almost always complete. Peptide fragments from all cleavable lysine positions were detected. Peptide fragments after amino acid position 97 were most often not detected by mass spectrometry. Interestingly, under the same digestion conditions, the digestion of α-syn adsorbed on 20 nm Au NPs depends strongly on the trypsin/α-syn ratios. Decreasing the trypsin/α-syn ratio increased the number of peptides detected (Figure 3.7). These peptide fragments were also longer, an indication that some lysine positions were not as accessible and hence not cleaved. With a trypsin/α-syn ratio of 1:160 (low trypsin ratio), most lysine positions at 21, 23, 32, 34, 79, 96, and 97 were already cleaved, implying that these positions were most possibly freely accessible to trypsin. Increasing the amount of trypsin (trypsin/α-syn 1:100) resulted in additional lysine positions at 43, 45, 58, and 60 being cleaved, as observed by the shorter and fewer peptide fragments on the Au NP surface, an indication of an increased rate of digestion with increasing amounts of trypsin. Further increases in the amount of trypsin (trypsin/α-syn 1:5) resulted in fewer and shorter peptide fragments on the Au NP surface. A comparison of the peptide fragments (in all three cases) detected in the supernatant and on Au NPs consistently showed the N-terminus (from amino acid 1 to 12) on the Au NP surface while being absent in the supernatant. Peptide fragments from amino acids 12 to 60 were also associated with the Au NP surface, whereas peptide fragments from amino acid positions larger than 60 were more often located in the supernatant.
Interestingly, changing the trypsin/\(\alpha\)-syn ratio did little to vary the digestion pattern of \(\alpha\)-syn on 90 nm Au NPs. A very similar pattern was observed for all three trypsin/\(\alpha\)-syn ratios where short peptide fragments ranging from amino acids 11 to 58 were found in the supernatant (Figure 3.8). Peptide fragments at amino acid positions larger than 60 were not detected in the supernatant. Similar to 20 nm Au NPs, the N-terminus was consistently found only on the 90 nm Au NP surface and was absent in the supernatant. However, unlike \(\alpha\)-syn on 20 nm Au NPs, only peptide fragments with an amino acid position not exceeding 21 were found on the 90 nm Au NP surface.

A comparison of digestion patterns of free \(\alpha\)-syn with bound \(\alpha\)-syn on 20 nm citrate-capped Au NPs reveals the following: (1) The first 12 amino acids of the N-terminus bind consistently to the Au NP surface, as denoted by the high proportion of the N-terminus peptides on the Au NP surface (and lack thereof in the supernatant). (2) Some regions of \(\alpha\)-syn are more accessible to trypsin than others, as shown by the longer peptide fragments on the Au NP surface with decreasing trypsin/\(\alpha\)-syn ratios. Varying the trypsin/\(\alpha\)-syn ratio altered the fragmentation pattern, indicating that certain parts of \(\alpha\)-syn are more protected than others. When the accessibility of trypsin to the lysine sites was plotted, a radical shift in the frequency of digestion sites was seen (Figure 3.5). On free \(\alpha\)-syn, the most digested lysine site is at position 80 whereas on 20 nm Au NP, positions 10, 21, and 23 are more heavily digested. (3) Peptide fragments on the Au NP surface, although less digested, follow a similar pattern to that of free \(\alpha\)-syn, signifying that the native unstructured morphology is maintained when absorbed onto Au NPs. In fact, it had been shown that for \(\alpha\)-syn fibrils the amino acid sequence from 32 to 102 is protected from trypsin digestion.\textsuperscript{55} It should be pointed out that because of the lack of lysines in the C-terminus, the fragment from amino acids 101 to 140 remains as a single peptide fragment and
Figure 3.8. Digestion pattern of α-syn adsorbed on 90 nm Au NPs at trypsin/α-syn ratios of (a) 1:160, (b) 1:100, and (c) 1:5. The peptide fragments in the supernatant are separated from those on the Au NP surface and are shown separately. Color scale (normalized) is the same for all plots.
was sometimes seen on the Au NP surface. However, because of limitations to the upper mass range on the mass spectrometer, such information remains inconclusive. In fact, when free α-syn was digested, the C-terminus fragment was not detected in all three ratios.

On the 90 nm Au NPs, similar to 20 nm Au NPs, the N-terminus was bound consistently to the Au NP surface. However, a more extensive digestion pattern was observed on 90 nm Au NPs, with few changes when the trypsin/α-syn ratio was changed. This high rate of digestion, coupled with the lack of changes, signifies that α-syn on 90 nm Au NPs was more susceptible to trypsin and thus more loosely packed and hence more exposed than on 20 nm Au NPs. This is reiterated by α-syn surface density analysis, where the surface density of 0.28 α-syn/nm² was obtained for 20 nm Au NPs and 0.21 α-syn/nm² on 90 nm Au NPs. These results are in line with DLS measurements that show that the hard corona on 90 nm Au NP is not as thick as on 20 nm Au NP (10 nm versus 15 nm) and hence is more amenable to trypsin digestion, which further signifies that some differences exist in the α-syn structure and orientation between 20 and 90 nm Au NPs.

On the basis of the amino acid sequence and structure of native α-syn, negatively charged, citrate-capped Au NPs would have a strong affinity for the N-terminus, in accordance with our digestion data and overall binding constants. The absence of N-terminus peptide fragments in the supernatant reflects the strong binding constants of α-syn bound to Au NPs, consistent with our data that suggests that the hard corona binds much more strongly to the gold surface than does the soft corona. However, the binding of α-syn to Au NPs is significantly different from that of lipid vesicles (Table 3.1). Au NP surfaces with citrate ion ligands present a relatively hard platform compared to lipid vesicles, which favor the formation of the α-helix at the N-terminus because the hydrophobic face of the α-helix can penetrate the lipid bilayer. (The
α-helical structure of α-syn is amphoteric; the hydrophilic and hydrophobic halves are separated by lysine residues. This also further suggests that it is the combination of electrostatic attraction and lipid ordering that is necessary for the formation of the α-helical structure of α-syn on lipid vesicles. The fibrillar structure (β-sheet) form of α-syn is also highly unlikely because of the lack of differences in the digestion pattern from free and bound protein (lysine positions 58 and 60 are always placed in a loop region, as opposed to lysine positions 43, 45, 80 and 96, which would lie in the plane of the β-sheet).

Overall, the current data suggest that α-syn maintains its native unstructured conformation when bound on Au NPs, with the N-terminus strongly adsorbed onto the Au NP surface. In its natively unstructured globular state, long-range intramolecular contacts are believed to exist, which makes α-syn more compact that a random coil. The NAC region (amino acids 85–95) is shielded by the C-terminus (amino acids 110–130), and C-terminus residues 120–130 interact with residues 105–115 as well as the region about amino acid 20 on the N-terminus. These interactions, hydrophobic between the C-terminus and NAC region as well as electrostatic with the N-terminus, protect α-syn from aggregation. The digestion of α-syn on Au NPs reflects a similar orientation: the binding of α-syn via the N-terminus also means that the C-terminus is projected outward from the Au NP. The C-terminus is thus the first to be cleaved and released into the supernatant. The NAC region (amino acids 61–95), shielded by the C-terminus and lying just above the N-terminus, was seen to be attached to the Au NP surface only through uncleaved lysine position 60. However, once cleaved, it is removed from the Au NP surface and into the supernatant.

The interaction of proteins with protein-sized nanoparticles is of fundamental importance as the number of potential uses of nanoparticles for in vivo applications increases. It is expected
that Au NPs in aqueous solution can physisorb biomolecules from a rich biological medium.\textsuperscript{18} We show here that a similar process also occurs when Au NPs are incubated with only one type of protein, \(\alpha\)-syn. It can thus be inferred that this multilayered adsorption is not a result of localized protein adsorption but is due to the nature of the protein (i.e., charge separation and structure). The separation of charges at the N-terminus (slight positive) and C-terminus (negative) and the propensity of \(\alpha\)-syn to acquire secondary \(\alpha\) or \(\beta\) structures from its natively unfolded form could contribute to the multilayered adsorption. The physisorption of wild-type (WT) \(\alpha\)-syn to Au NPs is thus a good starting point as a model system for understanding \(\alpha\)-syn (or other protein) affinities and interaction with protein-sized substrates such as NPs. The potential for nanoparticle-enabled therapy is also high, should protein adsorption to nanoparticles decrease the \(\alpha\)-syn’s propensity to misfold and accumulate as potentially toxic protofibrillar or fibrillar species.

The data suggest that the physicochemical nature of a nanoscale surface (lipid vesicles, small or larger gold nanoparticles) can tune the particle–protein binding constants and adsorbed structure and orientation of \(\alpha\)-syn. How nanoparticle-bound protein responds to an event that would initiate aggregation, compared to free protein, is a fascinating question that is under active investigation in our laboratory.

### 3.4. Conclusion

We have shown that \(\alpha\)-syn binding to 20 and 90 nm Au NPs occurs via at least a two-step absorption: a hard \(\alpha\)-syn corona with a strong binding constant \(K_a = (2.9 \pm 1.1) \times 10^9 \text{ M}^{-1}\) for 20 nm Au NPs, \(K_a = (9.5 \pm 0.8) \times 10^{10} \text{ M}^{-1}\) for 90 nm Au NPs) followed by a softer, thermodynamically unfavorable and kinetically driven corona, which is in constant exchange
with free $\alpha$-syn in solution. Quantitative analysis of $\alpha$-syn in the hard corona puts $360 \pm 70$ $\alpha$-syn on 20 nm Au NPs and $5300 \pm 700$ $\alpha$-syn on 90 nm Au NPs. The orientation of $\alpha$-syn was also deduced using a protease digestion method in which $\alpha$-syn was determined to adsorb onto Au NPs via the N-terminus, possibly in its native conformation. A slightly different $\alpha$-syn conformation was adopted on 90 nm Au NPs as compared to 20 nm Au NPs, which might be significant in future applications of nanoparticle-enabled therapy considering $\alpha$-syn’s high propensity to misfold.

3.5. Acknowledgements

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3.6. References


CHAPTER 4

Adsorption, Conformation and Orientation of \( \alpha \)-Synuclein on Cationic Gold Nanoparticles

4.1. Introduction

\( \alpha \)-Synuclein (\( \alpha \)-syn) belongs to the synuclein family which also consists of \( \beta \)- and \( \gamma \)-syn.\(^1\) \( \alpha \)-Syn is a small presynaptic protein that is found in relatively high abundance in the brain.\(^2\) While its function is still not fully understood, it is believed to play a regulatory role in modulating synaptic plasticity,\(^3\) controlling presynaptic vesicle pool size,\(^4\) releasing of neurotransmitter and recycling of vesicles.\(^5\) \( \alpha \)-Syn is a natively unstructured protein but can also form aggregates of \( \beta \)-sheet fibrils called amyloids. These protein aggregates form the main component of Lewy bodies, a class of intracellular inclusions that is highly characteristic of Parkinson’s disease.\(^6\) Recent studies have also demonstrated \( \alpha \)-syn involvement in Alzheimer’s disease, dementia with Lewy bodies and multiple system atrophy.\(^7\) This closely interwined relationship between neurodegenerative diseases and \( \alpha \)-syn implies that the disruption of protein homeostasis through its misfolding can be the cause of neuronal death.\(^6\) There is thus much motivation to study the function, folding kinetics and aggregation pathways of \( \alpha \)-syn. More specifically, the ability to control the conformation of \( \alpha \)-syn would allow relationships between structure and function to be deduced.

The 140-amino-acid-long \( \alpha \)-syn can be broadly divided into three regions: an amphiphilic N-terminus region (residues 1–60) with four imperfect 11-residue amino acid
repeats, each containing a conserved sequence (KTKEGV); a hydrophobic and highly amyloidogenic central domain (residues 61–95), also known as the non-amyloid component (NAC) region, which contain an additional three KTKEGV motifs; and an acidic C-terminus (residues 96–140) which is rich in proline residues and has no structural propensity.\(^8\) \(\alpha\)-Syn has a natively unstructured conformation in aqueous buffer, but can fold into various conformations depending on the environment and conditions.\(^7\) The N-terminus 11-mer repeat units can fold into an apolipoprotein-like class-A2 helix that mediates its binding to phospholipid vesicles, which increases \(\alpha\)-syn’s \(\alpha\)-helical content from \(\sim\)3% to over 70%.\(^9,10\) The curvature of vesicles can influence the resultant \(\alpha\)-helix that forms: a horseshoe \(\alpha\)-helix is maintained on 20–25 nm vesicles while on \(\sim\)100 nm vesicles an extended \(\alpha\)-helix is formed.\(^11\) In contrast, the hydrophobic NAC region is essential for \(\alpha\)-syn aggregation and \(\beta\)-sheet fibril formation. Studies have shown that free NAC peptide can seed amyloid formation, which forms the basis of Lewy bodies.\(^12\) In fact, comparison with \(\beta\)-syn shows that the lack of the middle section of the NAC region (residues 73–83) of \(\alpha\)-syn results in a lower propensity to form fibrils, and the removal of residues 71–82 abolish fibril assembly in \(\alpha\)-syn.\(^13\)

Protein adsorption onto nanoparticles (NPs) can alter the proteins’ conformation and influence its binding orientation. For example, fluorescence and FTIR results indicate that \(\beta\) -lactoglobulin’s tertiary structure changes over time when adsorbed on 90 nm silica NPs, while the secondary structure remained unchanged.\(^14\) In addition, the NP size and the protein’s size and stability also influence the proteins’ resultant conformational change when adsorbed onto silica NPs.\(^15–17\) Circular dichroism studies have shown that proteins in the soft corona (non-covalent, reversible interactions) of silica NPs are also conformational altered, which in turn alters their enzymatic activity.\(^18\) The orientation of adsorbed proteins onto NPs can be deduced from
protease digestion, and this method has been used to analyze the orientation of cytochrome c, RNase A, lysozyme and human carbonic anhydrase on 4–15 nm silica NPs.\textsuperscript{19,20}

Gold nanoparticles (Au NPs) have interesting physical and electronic properties, which allows for exciting potential therapeutics and biomedical applications such as drug delivery agents and gene transfection agents.\textsuperscript{21–24} However, to truly understand and take the potential uses of Au NPs to the next level, fundamental questions about their interactions with biological entities needs to be first answered. It has previously been shown that when silica NPs are introduced into biological medium, proteins bind non-specifically onto the silica NP surface, resulting in the formation of a protein corona.\textsuperscript{25} This can afford further complications as proteins trapped maybe less available to cells and can adversely affect cellular pathways. The influence of the protein corona does not just end with its adsorption: protein-protein interaction is also increased due to the high density of proteins on the NPs, forming a soft corona around the NP. The formation of this protein corona (hard and soft) also alters the surface properties of the NPs\textsuperscript{26} and it is this layer that interacts with cells.\textsuperscript{27,28}

The surface charge and chemistry of NPs can influence protein behavior. The diversity of protein size, structure and charge anisotropy profoundly influence their interaction with charged Au NPs.\textsuperscript{29–31} For example, bovine serum albumin (BSA, 66.5 kDa) is a negatively charged protein at pH 7 with an isoelectric point of 4.7.\textsuperscript{32} BSA can adsorb onto both anionic and cationic Au NPs,\textsuperscript{26} however the adsorption of BSA onto citrate Au NPs (anionic) at room temperature is enthalpy driven, while the adsorption of BSA onto cetyltrimethylammonium bromide Au NPs (cationic) is entropically favored.\textsuperscript{33} Upon adsorption to Au NPs, the secondary structure of BSA changes more significantly when positively charged Au NPs are used.\textsuperscript{33–35} In contrast, cytochrome C (CytC, \(\sim\) 12 kDa) is a positively charged protein at pH 7 with an isoelectric point
of ~10. The adsorption of CytC onto anionic Au NPs is an entropy controlled process, and involved CytC reorientation on Au NP surface at high concentrations. The binding orientation of CytC depends on the surface charge (anionic vs. cationic) as well as the degree of hydrophilicity (anionic) and hydrophobicity.

We have previously studied the interaction of α-syn with 20 and 90 nm anionic citrate-capped Au NPs. Our results showed that on negatively charged Au NPs, α-syn preferentially adsorbs via its N-terminus while retaining its natively unstructured conformation. In this chapter, we varied the surface charge of Au NPs using poly(allylamine hydrochloride) (PAH). Instead of forming a hard and soft corona, it was found that α-synuclein agglomerates on PAH Au NP and subsequently causes the Au NPs to flocculate out of solution. Using circular dichroism and in situ trypsin digestion, we investigate how the positively charged PAH Au NPs would influence α-syn’s conformation and orientation upon adsorption. Our results show that upon adsorption onto PAH Au NPs, α-syn’s conformation is altered but a preferred orientation cannot be distinguished. This work has great implications for the understanding of amyloid protein aggregation and potential avenues to control it.

4.2. Materials and Methods

4.2.1. Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium citrate tribasic dihydrate (Na₃Ct·2H₂O, ≥99%), poly(allylamine hydrochloride) (PAH, M.W. 15 000 g/mol) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥99.5%) were obtained from Sigma-Aldrich and were used as received. Sequencing-grade trypsin (lot 082301) and trypsin beads was obtained from GBioscience. Ultrapure deionized water (17.9 MΩ, Barnstead Nanopure II) was used for all solution preparations. A solution of HEPES buffer (20 mM, pH 7) was prepared in
ultrapure deionized water. Trypsin solutions were made by reconstituting trypsin in (NH₄)₂CO₃ (25 mM) to a final concentration of 12.5 μg/mL (0.54 μM). Glassware was cleaned with aqua regia and rinsed thoroughly before use.

4.2.2. Synthesis and Coating of Gold Nanoparticles

Au NPs (diameter 20 nm) were synthesized by the boiling citrate method as previously described with some modifications.³³ Briefly, 2.5 mL of 0.01 M HAuCl₄·3H₂O was added to 97.5 mL of ultrapure water in a 200 mL Erlenmeyer flask and heated to boiling. An aqueous solution of sodium citrate (5% w/w, 2 mL) was then added, and the solution was allowed to boil for another 30 min. During this period, the color of the solution slowly changed to deep red. Another portion of sodium citrate (1% w/w, 1 mL) was added, and the solution was allowed to boil for another 30 min. Au NPs produced by this method yielded a diameter of 17.5 ±1.3 nm under transmission electron microscopy (TEM). The hydrodynamic diameter measured by dynamic light scattering was 31.2 ± 0.4 nm.

Poly(allylamine hydrochloride) (PAH) Au NPs were made by first purifying 1 mL of citrate Au NPs by centrifugation. The Au pellet was collected and resuspended in 1 mL of water. To this purified Au NPs pellet, 100 μL of NaCl (0.1 M) and 200 μL of PAH (10 mg/mL) were added simultaneously, and the solution was vortexed. The NPs were allowed to incubate overnight before being purified by centrifugation.

4.2.3. Production of α-Synuclein

α-Syn protein was expressed and purified as previously described.³⁴ Briefly, the gene encoding wild-type human α-syn (SNCA) was cloned into the pET28 vector (Novagen). Protein
expression was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 37 °C, followed by alkaline lysis, boiling, and precipitation with 60% ammonium sulfate. Precipitates were resolubilized and purified by hydrophobic interaction chromatography on a HiPrep 16/10 butyl fast flow Sepharose column (GEBiosciences) and eluted with decreasing salt. α-Syn-containing fractions were pooled and concentrated, subjected to size-exclusion chromatography on a Superdex 75 10/300 GL column (GEBiosciences), and lyophilized for storage at −80 °C. The concentrations of α-syn solutions were determined by measuring the UV absorbance at 280 nm using an extinction coefficient of 5200 M⁻¹cm⁻¹.³⁵

4.2.4. Incubation of α-Synuclein with Gold Nanoparticles

Prior to each experiment, an aliquot of α-syn was reconstituted in HEPES buffer at a 1 mg/mL (70 μM) concentration. Various amounts of α-syn was then added to a concentrated volume of Au NPs such that α-syn had either saturated the Au NP surface or at a 100:1 α-syn:Au NP ratio. The α-syn bound Au NPs were incubated at 4 °C and allowed to equilibrate overnight. α-Syn saturated Au NPs was purified from excess free α-syn by centrifugation at least three times and dispersed in HEPES buffer.

4.2.5. Trypsin Digestion of α-Synuclein on Au NPs

To investigate the extend of protection on α-syn by Au NPs, fixed volumes of trypsin solution were added to known number of α-syn coated Au NPs such that the ratio of trypsin/α-syn ranged from 1:20 to 1:200. The samples were microwave digested for 15 min at 55 °C (70 W), following which separation was achieved using Amicon filters (MWCO = 30 000 Da) with centrifugation at 14,000 rcf for 5 min. The supernatant was collected, dried down, and analyzed
using nanoliquid chromatography mass spectrometry/mass spectrometry (LC MS/MS). The Au pellet was further purified twice before the digestion of the Au NPs with 1 M KCN, lyophilized to dryness, and analyzed.

For timed studies, α-syn bound Au NPs (α-syn:Au NP ratio 100:1, final Au NP concentration 20 nM) were heated in a heat block at 37 °C. Trypsin was added such that the resultant trypsin:α-syn ratio was 1:187. The reaction was quenched using formic acid (2%) at various time points. Separation was achieved using Amicon filters (MWCO = 30 000 Da) with centrifugation at 14,000 rcf for 5 min. The supernatant was collected, dried down, and analyzed using nanoliquid chromatography mass spectrometry/mass spectrometry (LC MS/MS). The Au pellet was further purified twice before the digestion of the Au NPs with 1 M KCN, lyophilized to dryness, and analyzed.

For reactions using trypsin beads, 50 µL of purified trypsin beads were added to α-syn bound Au NPs (50 nM, 600 µL). The sample was placed on a shaker at 37 °C and the reaction quenched with formic acid (2%) at various time points. Separation was achieved using Amicon filters (MWCO = 30 000 Da) with centrifugation at 14,000 rcf for 5 min. The supernatant was collected, dried down, and analyzed using nanoliquid chromatography mass spectrometry/mass spectrometry (LC MS/MS). The Au pellet was further purified twice before the digestion of the Au NPs with 1 M KCN, lyophilized to dryness, and analyzed.

The mass spectrometry data was processed using Waters Protein Lyns Global Server 2.2.5, Mascot (Matrix Sciences) and blasted against the NCBI-NR protein database for wild-type human α-syn (gi|4507109).
4.2.6. Circular Dichroism Measurement of α-Syn on PAH Au NPs

To prepare samples for circular dichroism measurements, solutions of 4 µM of α-syn were incubated overnight at 4 °C with 0, 1, 2, and 4 nM of 20 nm PAH-coated Au NPs in 10 mM MOPS buffer (pH 7). The sample housing in the CD was adjusted to 4 °C to maintain the incubation temperature. Samples were placed inside 1 mm quartz cuvettes (Starna Cells, Inc.). Each trace was an average of 10 scans, with integration time set as a function of photomultiplier voltage. The bandwidth was set at 8 nm. After collection, the data was processed with the Olis digital filter (size #11). Cuvettes were cleaned with aqua regia and rinsed thoroughly with water prior to each measurement. Secondary structure content was calculated through the SELCON3 fitting algorithm, reference set SP43.43

4.2.7. Instrumentation

A microcentrifuge (Eppendorf model 5418, Fisher-Thermo Electron) was used in various steps of synthesis and purification as detailed above. Protein digestion was performed on a CEM Discover microwave digestor (Mathews, NC). The mass spectrometer used for peptide analysis was a Waters quadrupole time-of-flight mass spectrometer (QToF) connected to a Waters Nano Acquity UPLC. A Waters Atlantis C-18 (0.03 mm particle, 0.075 mm × 150 mm) column was used, with the flow rate set to 250 nL/min. Peptides were eluted using a linear gradient of water/acetonitrile containing 0.1% formic acid 0–60% B in 60 min. The mass spectrometer was set for data-dependent acquisition; ms/ms was performed on the most abundant four peaks at any given time.
4.3. Results

4.3.1. Binding of α-Syn to Cationic PAH Au NPs

α-Syn is an amyloid protein which is prone to aggregation. To probe the propensity of α-syn to flocculate in the presence of PAH Au NPs, the overall binding of α-syn to PAH coated Au NPs was investigated using UV-vis spectroscopy and dynamic light scattering (DLS) (Figure 4.1). In UV-vis spectra, the plasmon band maxima is very sensitive to local refractive index changes around the Au NP,\(^4\) which can be induced by α-syn adsorption. When α-syn (0–14 µM) was titrated into 1 nM PAH Au NPs, the peak maxima was observed to shift a total of 18.3 nm (Figure 4.1a). This large shift in UV-vis maxima can be broadly characterized into two regions: a shift in peak maxima from 522.0 nm (0 µM α-syn) to 526.3 nm (2 µM α-syn) at lower α-syn/Au NP ratios (<2000:1), and a second increase in UV-vis spectra to a maximum of 540.3 nm (14 µM α-syn) at higher α-syn/Au NP ratios (>2000:1). While the peak maxima maintained its FWHM at lower α-syn/Au NP ratios, a significant broadening was observed at higher α-syn/Au NP ratios. Hydrodynamic diameter results from DLS measurements reflected similar trends (Figure 4.1b). At lower α-syn/Au NP ratios (<2000:1), hydrodynamic diameter increased from an initial of 31.2 ± 7.0 nm (0 µM α-syn) to 45.7 ± 10.5 nm (2 µM α-syn). Increasing α-syn/Au NP ratio further resulted in a bimodal Gaussian distribution of a smaller and larger Au NP population: the smaller population continued to increase slowly in hydrodynamic diameter from 83.3 ± 9.5 nm (3 µM α-syn) to 214.1 ± 78.1 nm (14 µM α-syn) while the hydrodynamic diameter in larger population increased exponentially from 191.3 ± 11.0 nm (4 µM α-syn) to 4860.8 ± 661.6 nm (14 µM α-syn).

The plasmon band shift of 4.3 nm from an initial 522.0 nm suggest suggests a significant change in the refractive index of 20 nm PAH Au NPs, and can be due to a very large α-syn
Figure 4.1. Titration of α-syn into PAH Au NP solutions. a) UV-vis spectral changes with increasing amounts of α-syn (0 –14 µM) added to 1 nM 20 nm PAH Au NPs. b) Change in absorption peak maxima of Au NPs as a function of α-syn concentration, as derived from (a). Gray region signifies α-syn/PAH Au NP ratio of >2000:1. c) Hydrodynamic diameter changes of 20 nm PAH Au NPs with increasing amounts of α-syn (0 –14 µM) added to 1 nM 20 nm PAH Au NPs. A bimodal distribution of sizes (red and blue) is observed after α-syn/Au NP >2000. d) Hydrodynamic diameter changes of PAH Au NPs as a function of α-syn concentration, as derived from (c). Gray region signifies α-syn/PAH Au NP ratio of >2000:1.
corona. The hydrodynamic diameter increase of 14 nm signifies that a multilayered adsorption of α-syn occurred (α-syn hydrodynamic diameter is 5.3 ± 1.0 nm45), as this increase is not large enough to account for nanoparticle dimers or trimers. In addition, visual observations of the flocculates showed that the sediments still retain their bright red color, characteristic of single Au NPs and not black aggregates, usually seen when Au NPs aggregate (Figure 4.2). The collective results suggested a very thick and possible multilayered α-syn corona formation on PAH Au NPs which subsequently cause the assemblies to agglomerate and fall out of solution (Scheme 4.1).

The tendency of α-syn coated PAH Au NPs to form bigger clumps and flocculate out of solution also suggested that α-syn is more ‘sticky’ and implies a conformational change (vide infra).

Due to the sensitivity of the plasmons in Au NPs to local refractive index change, the shift in UV-vis peak maxima and hydrodynamic diameter has often been used to deduce the binding affinity of proteins to Au NPs. Plotting the peak maxima shifts with respect to α-syn concentration showed a gradual shift in peak maxima (<2 µM), followed by an exponential increase (Figure 4.1b). Similarly, for the changes in hydrodynamic diameter, a gradual increase in hydrodynamic diameter (up to ~14.5 nm) was observed below 2 µM. At α-syn concentrations above 2 µM, both the small and larger flocculates were observed to continuously increase in size as α-syn concentration increases, with the larger flocculate size increasing at an exponential rate. Because the data do not show surface saturation of the protein (similar to α-syn adsorption on anionic Au NPs39), we were unable to calculate equilibrium binding constants.

We probed the extent of the dissociation of the outer layers by diluting PAH Au NPs sequentially after incubation with excess α-syn (α-syn/Au NP ratio 7000:1). Samples were diluted with buffer such that the final concentration of α-syn was reduced from 7 µM to 4.6, 3.5
Figure 4.2. Flocculation of Au NPs. (Left) Agglomeration of α-syn coated PAH Au NPs (α-syn/PAH Au NP 4000:1). The agglomerate remained red in color and can be resuspended by pipetting. (Right) Aggregation of citrate Au NPs in a high salt environment. The aggregates are black in color and cannot be resuspended as a red suspension again.
Scheme 4.1. The Proposed Adsorption of α-Syn onto PAH Au NPs.$^a$

At low α-syn/PAH Au NP ratio, α-syn adsorb as multilayers while at high ratio, the α-syn coated PAH Au NPs agglomerates. The different colors of free and bound α-syn suggest a possible conformation change.
and 2.3 µM (Figure 4.3a). However, even after 3x dilution to 2.3 µM α-syn, no significant change in the hydrodynamic diameter was observed. A bimodal distribution at around 212.3 ±24.3 nm and 841.8 ± 101.0 nm was still seen, similar to the undiluted sample (7 µM α-syn) at around 309.8 ± 48.8 nm and 1011.6 ± 148.8 nm. A shift to larger hydrodynamic diameter for both small and large flocculate populations was instead observed even when α-syn coated PAH Au NPs were incubated at 4 °C for longer time periods (Figure 4.3b). At 4000:1 α-syn/Au NP ratio, the hydrodynamic diameter obtained after 1 day was 84.1 ± 4.5 nm and 191.3 ± 11.0 nm, which increased to 100.7 ± 10.0 nm and 409.8 ± 42.6 nm after 2 days. The hydrodynamic diameter further increased to 129.0 ± 18.6 nm and 1307.6 ± 201.7 nm after 7 days incubation.

4.3.2. Conformation and Orientation of α-Syn on a Saturated PAH Au NP Surface

Circular dichroism (CD) is commonly used to detect changes in protein conformation as a function of an external factor,46 and has been used to detect protein conformation changes on Au NPs.47,48 Here, we use CD to observe changes in the secondary structure of α-syn upon interaction with PAH coated Au NPs after an overnight incubation at 4 °C. The molar ratio of α-syn to PAH Au NPs in this experiment was maintained such that α-syn was always in excess to Au NPs (1000–4000 α-syn/Au NP). The CD spectrum of native α-syn is characterized by its unstructured conformation (Figure 4.4). Addition of Au NPs caused a decrease in signal at around 200 nm, from -14.1 m° for α-syn without Au NPs, to -11.2, -11.1 and -11.5 m° when PAH Au NPs was added at 1, 2 and 4 nM respectively. Quantitative analysis of the spectrum shift was performed from 200 - 250 nm using a SELCON3 fitting algorithm (Table 4.1).43 The CD spectrum of free α-syn was fitted to 14% α helical, 7% β sheet, and 37% unstructured conformation. Upon addition of PAH Au NPs, regardless of PAH Au NP concentration, α helical
Figure 4.3. a) Dynamic light scattering (DLS) plots showing bimodal size distribution of \( \alpha \)-syn coated PAH Au NPs after a series of dilutions with HEPES buffer from an initial \( \alpha \)-syn concentration of 7 µM (black), to 4.6 µM (red), 3.5 µM (green) and 2.3 µM (blue). The concentration of PAH Au NP is 1 nM. b) DLS plots showing the aggregation of \( \alpha \)-syn coated PAH Au NPs after 1 day (black), 2 days (red) and 6 days (green) of incubation at 4 °C. \( \alpha \)-Syn/PAH Au NP ratio is 4000:1. DLS data is fitted to Gaussian distributions and are plotted as the mean ± one standard deviation.
Figure 4.4. Circular dichroism spectra of α-syn adsorbed on PAH Au NPs. The concentration of native α-syn (MOPS buffer, 10 mM, pH 7) was kept at 4 µM (black). Au NPs was added for a final concentration of 1 nM (red), 2 nM (green), 4 nM (blue) (α-syn/Au NP ratio of 4000:1, 2000:1 and 1000:1 respectively).
Table 4.1. Percent of Secondary Structures as Deduced from the Circular Dichroism Spectrum of α-Syn Incubated With and Without PAH Au NPs.\textsuperscript{a}

<table>
<thead>
<tr>
<th>[AuNP] (nM)</th>
<th>α-helix (%)</th>
<th>disordered α-helix (%)</th>
<th>β-sheet (%)</th>
<th>disordered β-sheet (%)</th>
<th>turn (%)</th>
<th>unordered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14</td>
<td>13</td>
<td>7</td>
<td>7</td>
<td>16</td>
<td>37</td>
</tr>
<tr>
<td>1.0</td>
<td>6</td>
<td>11</td>
<td>29</td>
<td>12</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>2.0</td>
<td>6</td>
<td>12</td>
<td>28</td>
<td>12</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>4.0</td>
<td>6</td>
<td>11</td>
<td>28</td>
<td>12</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Secondary structure content was calculated using SELCON3 fitting algorithm. α-Syn = 4 μM.
and unstructured content was decreased from 14% to 6%, and 37% to 20%, respectively, while β sheet conformation was increased from 7% to 29%. The lack of a concentration dependent change in CD spectra suggests that PAH Au NPs can induce a change in conformation of bound α-syn, which is then propagated to α-syn not in direct contact with PAH Au NP surface (vide intra).

To deduce the orientation of α-syn on PAH Au NPs, an in situ trypsin digestion method was used (Scheme 4.2). Excess α-syn was adsorbed onto PAH Au NPs and allowed to incubate overnight at 4 °C. After purification via centrifugation to remove unbound α-syn, trypsin was added to α-syn coated PAH Au NPs and microwave digested. Trypsin digests α-syn at lysine positions (α-syn does not have arginine, another site of trypsin digestion). A total of 15 lysine positions in α-syn are available for cleavage: at positions 6, 10, 12, 21, 23, 32, 34, 43, 45, 58, 60, 80, 96, 97 and 101. The released peptide fragments were then separated from the peptide fragments still bound on Au NPs and quantified using mass spectrometry (MS), from which the orientation of α-syn can be deduced. First, by comparing the peptide fragments released into solution with that still bound on PAH Au NPs, it is possible to draw conclusions about the peptides which had a higher affinity for PAH Au NPs, hence deduce α-syn orientation. Secondly, compared to the free α-syn digestion pattern, lysine positions that were not as readily cleaved are more protected, suggesting limited trypsin accessibility. This is reflected by the larger number of longer, overlapping peptide fragments with missed cleavages. Thirdly, the relative proportions of peptide fragments on changing the trypsin/α-syn ratio reveal the extent of protection.

Figure 4.5 shows the digestion pattern for free α-syn, α-syn in the presence of PAH polymer and α-syn adsorbed on PAH Au NPs. The number of peptides was normalized with respect to the highest peptide count in each experiment for ease of comparison and is shown as a
Scheme 4.2. Cartoon of Au NP Coating with PAH and the In Situ Trypsin Digestion Method Used to Deduce α-Syn Orientation

Note that the horseshoe structure of α-syn shown here is not representative of its actual conformation on PAH Au NPs.
Figure 4.5. Digestion pattern of a) free α-syn, b) α-syn incubated with PAH polymer and α-syn incubated with PAH Au NPs (8.3 pmoles) at trypsin/α-syn ratios of (c) 1:200, (d) 1:100, and (e) 1:20. The peptide fragments in the supernatant are separated from those on the Au NP surface and are shown separately. The horizontal axis denotes the amino acids in α-syn from the N to C terminus. The vertical axis denotes the different peptide fragments obtained, which is further separated into those in the supernatant and on the Au NP surface. The color scale (normalized) denotes the number of peptides found. The scale on the z axis is the same for all plots.
color scale in the z-axis. The peptide fragments are arranged according to their amino acid positions on the x-axis and the y-axis tabulates the all the peptide fragments detected. Results showed that for α-syn adsorbed on PAH Au NPs, peptide fragments found on Au NP surface were very similar to those in the supernatant; peptide fragments from the full α-syn sequence was detected in both solutions. Similar to free α-syn and α-syn in the presence of PAH polymer, peptide fragments 1–6, 61–79 and 80–96 were present in high proportions. However, it should be noted that while peptide fragment 81–96 was of the highest frequency in free α-syn, the peptide fragment with highest frequency on PAH Au NP is 1–6. Comparison of α-syn incubated with PAH polymer showed a similar trend: peptide fragment 1–6 was of the highest frequency, implying that the change in frequency can be due to the interaction of α-syn with PAH polymer.

The number of missed cleavages (which translates to longer peptide fragments) at lysine positions 23, 34 and 60 were less often observed when α-syn was absorbed onto PAH Au NPs. In contrast, longer peptide fragments at these locations can be seen from the digest of α-syn with and without PAH polymer.

The extent of protection of α-syn on PAH Au NPs was studied by varying the trypsin/α-syn ratio at 1:200, 1:100 and 1:20 (Figure 4.5). Results showed that the digestion pattern was not altered significantly on varying trypsin/α-syn ratio. In particular, the number of peptide fragments in all three ratios and their frequencies of detected were similar. All peptide fragments were detected in both the supernatant as well as on Au NP surface.

4.3.3. Digestion of α-Syn on a Non-Saturated Au NP Surface

The propensity of α-syn-coated-PAH Au NPs to aggregate made quantification of peptides after trypsin digestion extremely challenging. To overcome this problem, a reduced α-syn coverage of 100:1 (α-syn/Au NP) was used instead of saturation conditions. This
circumvents the problem in two ways: 1) flocculation is avoided and 2) the need to separate out free (unbound) α-syn is eliminated. For comparison, α-syn was incubated on both citrate (anionic) and PAH (cationic) Au NPs.

Trypsin was added to α-syn samples to achieve a resultant 1:187 ratio and digested at 37 °C for 8–16 h. Figure 4.6a and b shows the peptide fragments obtained in the supernatants and on citrate Au NPs or PAH Au NP surfaces. When α-syn on citrate Au NPs was digested, very few of the peptide fragments were released into the supernatant. In addition, only the N-terminus was found on the Au NP surface and a large fraction of the peptide fragments had an uncleaved lysine position 6 (peptide fragment 1–10). In comparison, when α-syn was absorbed on PAH Au NPs, the number of peptide fragments in the supernatant was similar to that found on the PAH Au NP surface. However, the respective ratio of peptide fragments is drastically different in the supernatant or on the Au NP surface. Peptide fragment 1–10 was more commonly found on PAH Au NP surface while peptide fragment 35–43 was more often in the supernatant.

The number of peptide fragments was tabulated to provide a more representative picture of the digestion pattern (Figure 4.6c). For ease of comparison, peptide fragments were grouped according to α-syn’s amino acid sequence: residues 1–23, 24–43, 44–58 and 59–97, where the first 3 groups reflects the N-terminus and the last group the non-amyloid component (NAC) region. The C-terminus residues 98–140 was excluded due to limitations on the detection limit of the mass spectrometer. We find that digestion of free α-syn resulted in the release of peptides from 1–23 and 59–97 at (35.2 ± 5.8) % and (36.5 ± 5.2) % of the total peptides detected respectively, while peptides with amino acids from 24–43 and 44–58 were found at lower ratios ((13.7 ± 1.0) % and (14.6 ± 1.6) % respectively). When α-syn was bound to citrate Au NPs, a significant increase in the fraction of peptides from 1–23 was observed at (76.4 ± 3.2) %, while
Figure 4.6. Digestion pattern of α-syn using trypsin when absorbed onto a) citrate Au NPs and b) PAH Au NPs. A α-syn/Au NP ratio of 100:1 was used. The peptide fragments in the supernatant are separated from those on the Au NP surface and are shown separately. Color scale (normalized) is the same for all plots. c) Fraction of peptide fragments from α-syn digestion when free in solution (black), absorbed on citrate Au NPs (red) and absorbed on PAH Au NPs (blue). Peptides were grouped into 4 groups according to their amino acid positions: 1–23, 24–43, 44–58 and 59–97. d) α-Syn lysine susceptibility to trypsin digestion when free in solution (black), absorbed on citrate Au NPs (red) and absorbed on PAH Au NPs (blue).
peptides from 24–43, 44–58 and 59–97 adds up to only about 23 % of the total peptides detected. Similarly, the fraction of peptides from 1–23 for α-syn on PAH Au NPs was high at (56.4 ± 7.0) %. However, the fraction of peptides from 24–43 and 44–58 of α-syn on PAH Au NPs were more similar to free α-syn, at (19.4 ± 4.2) % and (17.6 ± 3.3) % respectively, while peptide from 59–97 only made up (6.6 ± 1.9) % of the total peptide fragments.

The susceptibility of α-syn to trypsin digestion was mapped by illustrating the lysine positions which are susceptible to trypsin attack (Figure 4.6d). It is assumed that every peptide fragment is independent of each other: every peptide fragment is counted with two lysine cleavages (except terminal peptides) and tabulated. Since there are no lysine positions in the C-terminus, only the N-terminus and NAC region is considered (amino acids 1–101). Results show that after 2 h digestion, lysine at position 10 is more digested on both citrate and PAH Au NPs as compared to free α-syn while the accessibility at lysine position 6 is relatively similar. In contrast, lysine at position 80 is more digested with free α-syn than when absorbed on citrate or PAH Au NPs. Differences in the digestion of α-syn was detected between lysine positions 32 and 60, specifically at positions 34, 43 and 58. In this region, digestion on citrate Au NPs is suppressed, while on PAH Au NPs the rate of digestion is higher than free α-syn at lysine positions 34 and 43 and similar at position 58.

4.4. Discussion

While many studies have focused on investigating the adsorption, orientation and conformation of proteins with known tertiary structure on surfaces, the natively unstructured conformation of α-syn and its potential to aggregate implies a different adsorption kinetics when bound onto nanoscale surfaces. Indeed, α-syn coated Au NPs have been used to align Au NPs in amyloid fibrils, suggesting conformational changes of bound α-syn. In
addition, while the influence of NPs on the aggregation kinetics of amyloid proteins has been studied, this complicated process is still little understood. The study of \( \alpha \)-syn adsorption onto Au NPs is thus a good case study which can be extended to other unstructured and aggregation prone proteins.

We would like to highlight that while we used the more general term ‘flocculation’ to describe the instability of the \( \alpha \)-syn coated PAH Au NPs, in actual fact, these Au NPs agglomerate rather than aggregate. To allow readers a better understand the chemistry behind \( \alpha \)-syn coated PAH Au NPs, we paraphrase the definitions from Ross and Morrison, as well as from Weisbecker et al. A ‘floc’ is defined as any close association of primary particles, aggregates and agglomerates, and ‘flocculation’ is the process of forming them. ‘Aggregates’ are more closely associated particles and cannot be separated once they are formed, while ‘agglomerates’ are loose, reversible association of particles. Visually, \( \alpha \)-syn coated PAH Au NPs form red agglomerates which settled to the bottom of the suspension over time, and can be resuspended by pipetting (Figure 4.2). In contrast, citrate Au NPs aggregates when placed in a high salt environment to a purple/black suspension and will not revert to a red suspension even with sonication. For subsequent discussion, we will use ‘agglomerate’ to describe the instability of \( \alpha \)-syn coated PAH Au NPs.

At \(<2000 \ \alpha\text{-syn}/\text{PAH Au NP ratio}, a steady red shift in UV-vis (522.0 nm to 526.3 nm) and an increase of hydrodynamic diameter was observed (31.2 ± 7.0 nm to 45.7 ± 10.5 nm). This change in UV-vis and hydrodynamic diameter is characteristic of protein adsorption onto Au NPs. Given the hydrodynamic diameter of \( \alpha \)-syn is 5.3 ± 1.0 nm, DLS data suggest that the protein layer is about 1.5 times \( \alpha \)-syn’s hydrodynamic size. At \( >2000 \ \alpha\text{-syn}/\text{PAH Au NP}, \alpha\text{-syn coated PAH Au NPs continue to agglomerate and grow in size even when not agitated and}
placed in 4 °C. At 3000 α-syn/PAH Au NP ratio, the UV-vis peak maxima of 527.3 nm suggest a NP diameter of ~40 nm. A doubling of the hydrodynamic diameter was also seen on transiting from 2000 to 3000 α-syn/PAH Au NP ratio: 45.7 ± 10.5 nm to 83.3 ± 9.5 nm. These agglomerates, once formed, did not decrease in size when diluted. In addition, the agglomerates were observed to grow in size over time, suggesting that α-syn coated PAH Au NPs are extremely ‘sticky’.

The results suggest that the multilayered adsorption of α-syn onto PAH Au NPs does not make up the soft corona. The soft corona is usually defined as a labile, weakly interacting protein layer that is in constant exchange with proteins in solution, which can be further weakened by decreasing the probability of proteins colliding with NPs, i.e. dilution. However dilution does not cause the hydrodynamic diameter to decrease, indicating that the multilayered adsorption of α-syn is strong.

The increased tendency of α-syn adsorbed onto PAH Au NPs to agglomerate suggest a possible α-syn conformation change. The aggregation of free α-syn can be induced with agitation and heating to 37 °C. Under these conditions, aggregation is only often seen after 8 days (21 µM, 0.025 M Tris-HCL, pH 7.5, 37 °C). In the presence of spermine, a positively charged polyamine, aggregation kinetics is increased to a aggregation half-time of ~17 h (10 µM spermine, 1.75 µM α-syn, 25 mM Tris-HCL, pH 7.5, 37 °C). Previous studies have showed that positively charged polyamine such as spermine can bind to the C-terminus of α-syn, releasing the long range interactions with the N-terminus and opening up the compact α-syn structure. In our experiments, incubation of α-syn (< 14 µM) with PAH Au NPs at 4 °C without agitation induced agglomeration of PAH Au NPs, while with anionic citrate Au NPs, such agglomerations are not seen. The positively charged PAH Au NPs could have a similar interaction with α-syn:
binding of α-syn onto PAH Au NPs weakened the long range interactions such that α-syn has an increase affinity to aggregate, thus implying a conformational change of bound α-syn.

The agglomeration of α-syn coated PAH Au NPs made fitting of UV-vis and DLS data to a BET adsorption isotherm (or any isotherms) unreliable: the sensitivity of the UV-vis plasmon peak of Au NPs to protein adsorption is only accurate if the Au NPs are well separated from each other. In this case, it is usually assumed that the plasmon peak shift is due to the change in refractive index around the Au NP, which is caused only by protein binding. However, when α-syn coated Au NPs agglomerates, the UV-vis plasmon band shift is also now partially attributed to the inter-NP interactions. Similarly for DLS data, the increase in hydrodynamic diameter due to PAH Au NP agglomerates cannot be isolated from the increase due to α-syn adsorption.

To probe the orientation and potential long range interaction changes of α-syn on PAH Au NPs, we performed in situ trypsin digestion of α-syn when absorbed onto either citrate or PAH Au NPs. We have previously studied the binding of α-syn onto negatively charged citrate Au NPs.39 A hard α-syn corona comprising of strong electrostatic interactions and a soft corona of weaker noncovalent protein-protein interactions of α-syn on citrate Au NPs was observed.39 In addition, the orientation of α-syn binding onto citrate Au NPs was determined to be via the N-terminus, while the conformation remained unchanged. Digestion of α-syn on a saturated PAH Au NP surface showed that the full range of peptide fragments can be found in both the supernatant and Au NP surface, with the relative ratio of peptide fragments similar in each section. This further confirms the presence of multilayered α-syn adsorption on PAH Au NPs. The reduced number of missed cleavages implied that the peptide fragments were more readily digested; α-syn on PAH Au NPs were more accessible to trypsin attack than free α-syn, suggesting that some of the long range interactions were disrupted and hence α-syn is more
‘accessible’ to trypsin. Qin et al. had previously shown that when α-syn fibrils were subjected to trypsin digestion, residues 32–102 were protected from digestion.\textsuperscript{64} The possible on-particle formation of α-helical structure is unlikely: the hydrophobic side of the amphiphilic α-helix is unlikely to be stabilized on the hard PAH Au NP surface.\textsuperscript{65} Taken together, this suggest that while α-syn maintained its native unstructured conformation when binding to PAH Au NPs, this conformation is slightly altered such that it is more accessible to trypsin attack.

Further confirmation was provided by CD measurements: incubation of α-syn with PAH Au NPs increased the percentage of β-sheet structures and decreased the α-helical content, suggesting that PAH Au NPs can result in a change of secondary conformation in α-syn when adsorbed onto PAH Au NPs. It should be highlighted that this change in conformation is similar regardless of PAH Au NP concentration. In most cases, protein’s conformation changes are Au NP concentration dependent; increasing Au NP concentration decreases the α-helical content of bovine serum albumin.\textsuperscript{35} In our case, the lack of a concentration dependent change suggests that PAH Au NPs exert a global influence on α-syn; all (or almost all) of α-syn’s conformation was altered. In light of UV-vis and DLS data, which showed that α-syn coated PAH Au NPs agglomerate at high α-syn/PAH Au NP ratio, it is possible that the adsorption of the first α-syn layer on PAH Au NPs resulted in some conformational change, which as a result made bound α-syn more ‘sticky’, hence can attract more α-syn to bind which are also induced conformational to change. In contrast, α-syn adsorption onto negatively charged citrate Au NPs did not induce any observable conformational change in CD spectra\textsuperscript{39}, highlighting that α-syn maintained its compact, unstructured nature when adsorbed (long range interactions not disrupted). While changes in conformation has been shown to be inducible by nanoparticle size\textsuperscript{16,18,19,66}, we show
here that α-syn’s conformation on nanoparticles also depend on the nanoparticles’ surface charge and the change is propagated throughout the solution.

The multilayered adsorption of α-syn onto PAH Au NPs made it difficult to deduce the orientation of the bound α-syn. We thus studied the trypsin digestion of α-syn on a non-saturated Au NP surface. Since the conformation of α-syn remained in its unstructured conformation under saturated conditions for citrate Au NPs and consistently changed for PAH Au NPs, we believe that at unsaturated conditions, the conformation and orientation of α-syn on citrate and PAH Au NP remained similar to saturated conditions. We chose here to use a 100:1 α-syn/Au NP ratio based on previous experiments which showed that 360 ± 70 α-syn absorbs per citrate Au NP in the hard corona.39

Results showed that peptide fragments from the N-terminus are only found on the citrate Au NP surface, confirming that α-syn binds via the N-terminus on citrate Au NPs. In contrast, the digestion pattern of α-syn on PAH Au NPs once again displayed all peptide fragments in both supernatant and Au NP surface, suggesting that α-syn is randomly absorbed onto PAH Au NPs, i.e. there is no specific orientation. This can be explained by examining the conformation of the native unstructured α-syn: it is believed that the NAC region is shielded by the N- and C-terminus, with long range interactions between amino acid 120 on the C-terminus and amino acid 20 on the N-terminus.62 The N- and C-terminus are thus outward facing and can interact with Au NPs. The C-terminus (residues 96–140) is high in acidic amino acids, which can be strongly attracted to the positively charged PAH Au NP surface. The hydrophilic portion of the amphiphilic N-terminus (residues 1–65) is made up of both acidic and basic amino acids, and thus is not immune to absorption onto PAH Au NPs, although its interaction might be weaker.
By grouping peptide fragments according to their amino acid sequence, the section of α-syn which is more accessible to trypsin can be mapped. The lysine susceptibility plots also provided an idea of the ‘activity’ at a particular lysine site, which suggests that the adsorption of α-syn onto Au NPs increases its susceptibility to trypsin at the N-terminus (amino acid 1–23) while at the same time decreasing digestion at the NAC region (amino acid 59–97). These results can be explained by taking into considerations the long range interactions within α-syn, possibly additional binding sites to Au NPs as well as the close proximity of bound α-syn to each other on Au NPs. The intra-protein interactions are sufficiently strong, such that α-syn is composed of a more compact ensemble than would be expected of a random coil. Atomic force measurements demonstrated that the intra-protein interactions have an average of 64 ± 30 pN (10 mM Tris-HCl, pH 7.5), while inter-protein interactions between α-syn dimers required 43.1 ± 1.8 pN for separation. It is thus highly likely that when α-syn is bound on Au NPs, after trypsin digestion, peptide fragments are still held in close proximity to each other by the intra-protein and inter-protein interactions. These peptide fragments would still prevent trypsin from penetrating the inner core region of α-syn (lysine position 80). With free α-syn, trypsin digestion cleaves α-syn and the peptide fragment is able to move/diffuse away, allowing trypsin to further attack ‘protected’ sites. The increased accessibility of α-syn on PAH Au NPs at lysine positions 34, 43 and 58 might be due to α-syn’s random orientation on PAH Au NPs: when bound via the C-terminus, the change in conformation as a result of weakened intra-protein interactions might open up α-syn structure, allowing trypsin attack.

The lysine accessibility plot also highlights the differences at lysine position 10. On free α-syn, peptide fragments 1–10 can be further digested by trypsin to peptide fragments 1–6. Peptide fragment 7–10 is not detected as the peptide mass is not above the detection threshold.
Since it has been established that the absorption of α-syn on citrate Au NPs occurs via the N-terminus while on PAH Au NP it is randomly orientated, the increase digestion at lysine position 10 only serves to reinforce the idea that α-syn remains in its native conformation when bound. Assuming that α-syn is 4 nm in diameter and maintains a spherical shape, about 10 amino acids would be in contact with the Au NP surface. This estimate suggests that lysine at position 10 would be more exposed to trypsin than at position 6 when α-syn is absorbed on citrate Au NPs and for some of the α-syn on PAH Au NPs. Since the N-terminus peptides are bound onto Au NP surface, they cannot be further digested to the shorter peptide fragment (1–6), resulting in the larger amount of peptide fragments with amino acids 1–10.

Comparison of the digestion pattern of α-syn on both saturated and non-saturated citrate and PAH Au NP revealed differences in the accessibility of α-syn to trypsin (Figure 4.7). On the saturated citrate Au NP surface, the most preferred lysine digestion site was at position 21, with positions 10, 12, 43, 58 and 80 less preferred. However, on the non-saturated citrate Au NP surface, the most preferred digestion site was position 10. This difference in digestion pattern reflects the change in packing density of α-syn on either saturated or non-saturated citrate Au NP surface; the tighter packing on a saturated surface would limit trypsin accessibility to the N-terminus and hence position 10. On saturated PAH Au NP surface, α-syn is more digested at position 6 and 80, and less digested at positions 10, 43, 58 and 60, while on non-saturated PAH Au NP surface the preferred digestion site is at position 10. By taking into account the random orientation and the multilayered adsorption of α-syn on transiting from non-saturated to saturated PAH Au NP conditions, it can be observed that the digestion pattern of α-syn on saturated PAH Au NPs is a combination of α-syn on non-saturated PAH Au NPs and free α-syn in solution.
Figure 4.7. α-Syn amino acid sequence and the preferred digestion sites of a) free α-syn, b) α-syn on citrate Au NPs (saturated), c) α-syn on PAH Au NPs by trypsin (saturated), d) α-syn on citrate Au NPs (non-saturated), e) α-syn on PAH Au NPs by trypsin (non-saturated). On saturated Au NP surface, the trypsin/α-syn ratio is 1:100. On non-saturated Au NP surface, the α-syn/Au NP ratio used is 100:1 and the trypsin/α-syn ratio is 1:187. Lysine positions are highlighted in red and the frequency of trypsin attack is shown as arrows with a color scale, with black being the easiest for trypsin to attack and light gray being the least digested. Data from α-syn on citrate Au NPs (saturated) was obtained from Ref 39.
4.5. Conclusion

In conclusion, our results highlights that the surface chemistry of Au NPs is crucial in influencing α-syn binding. By titrating α-syn to positively charged PAH Au NPs, it was found that α-syn adsorbed in a multilayered fashion. The adsorption of α-syn onto PAH Au NPs altered its conformation, resulting in a consistent decrease in α-helix and an increase in β-sheet regardless of PAH Au NP concentration. The resultant α-syn coated PAH Au NPs are more ‘sticky’, and above 2000:1 α-syn/PAH Au NP ratio, tend to agglomerate into large flocculates and crash out of solution. Trypsin digestions illustrated the difference in α-syn orientation on either citrate or PAH Au NPs: on citrate Au NPs, α-syn adsorbed via the N-terminus while on PAH Au NPs, α-syn binds in a random orientation. Digestions obtained using non-saturated α-syn conditions showed similar trends. In addition, it was found that the fraction of peptides from amino acid 1–23 increased while that of 59–97 decreases upon adsorption to either citrate or PAH Au NPs when compared to free α-syn. By tabulating the ‘activity’ at each lysine position of α-syn, position 10 was more digested and 80 less digested when on either citrate or PAH Au NPs when compared to free α-syn. These results highlight that the accessibility of α-syn to trypsin is altered when adsorbed onto Au NPs. Our work on α-syn binding onto PAH Au NPs contributes to better understanding of nanoparticle mediated therapy for amyloid diseases.

4.6. Acknowledgements

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4.7 References


CHAPTER 5

Tuning Cellular Response to Nanoparticles via Surface Chemistry and Aggregation*

5.1. Introduction

The excellent synthetic size and shape control of gold nanoparticles (Au NPs) allows for the localized surface plasmon resonance (LSPR) of Au NPs to be easily fine-tuned. Coupled with the ease of surface functionalization of Au NPs, the use of Au NPs is very popular in biological applications.1–4 For example, the sensitivity of the LSPR to changes in the Au NPs’ immediate environment as well as the high scattering coefficient of metallic Au NPS allows them to be used as sensing and imaging probes.5–7 The conversion of absorbed light into heat by Au NPs shows potential in photothermal therapy.8,9 Small molecules can also be attached via various means (electrostatic, covalent, hydrophobic) to Au NPs in order to enable applications in gene/drug delivery.10,11 However, while Au NPs have the potential to revolutionize nanotechnology based biomedical applications, in order to predict their biological interactions in detail, a more holistic understanding of the particle physiochemical properties after immersion in biological media is required.

In a usual in vitro cell-nanoparticle experiment, cells are first plated on a hard substrate such as a culture dish. The Au NPs are then dispersed in cell media, which is added as a suspension to the cells. In such a scenario, the Au NPs are often assumed to be well-dispersed.

* This chapter has been submitted to Small as: Jie An Yang, Samuel E. Lohse, Catherine J. Murphy. ‘Tuning Cellular Response to Nanoparticles via Surface Chemistry and Aggregation.’
However, Au NPs are commonly observed to aggregate when introduced into a high protein, high salt environment.\textsuperscript{12} Au NPs aggregation is also observed as a function of pH and results from the presence of cross linking species (e.g., divalent cations or polyelectrolytes) in solution, which implies that NPs in cellular or environmental media are primarily stabilized electrostatically.\textsuperscript{13} Accordingly, NP aggregation complicates \textit{in vitro} studies of NP bio-interactions. For instance, \textit{in vitro} uptake of aggregated Au NPs has been shown to occur at different rates than well-dispersed Au NPs (although the precise uptake rate also depends on the initial Au NP size and the cell type).\textsuperscript{14,15} In addition, metallic Au NPs are dense and can settle to the bottom of the culture plates over time, and such phenomena has been observed for Au NPs larger than 90 nm in diameter as well as long gold nanorods (Au NRs).\textsuperscript{16,17} This poses another challenge as NP uptake by cells is directly related to NP concentration, and cells plated at the bottom or top of the culture plate will have different cellular uptake of NPs as the cells experience a different effective NP dose.\textsuperscript{12,17,18}

Even before introduction of NPs to cells, evidence points towards the formation of a protein corona around NPs when incubated with cell media or blood plasma.\textsuperscript{19,20} The adsorption of proteins occurs regardless of initial surface chemistry on NPs (although surface chemistry may influence the composition of the corona). Au NPs with different initial surface charges have been shown to develop similar $\zeta$-potentials after incubation in cell media loaded with bovine serum albumin (BSA), signifying BSA adsorption.\textsuperscript{21} The adsorption of proteins onto NPs is non-specific: a plethora of proteins bind onto the NP surface when introduced into blood plasma.\textsuperscript{22} Further analysis of the protein adsorption onto NPs showed that protein adsorption can be resolved into two layers: a hard inner corona of strongly associating proteins and a soft outer corona comprised of weakly bound, highly labile proteins in constant exchange with free
proteins in solution, which ultimately influences how cells “perceive” NPs.\(^ {23,24}\) While this protein corona had been shown to mitigate and reduce NP’s capabilities and functions\(^ {25}\), effects from NP incubation is still felt by cells. For example, cetyltrimethylammonium bromide (CTAB), a surfactant that is commonly used in the synthesis of Au NPs and is toxic to certain culture cells at 10\(^{-7}\) M concentrations\(^ {21}\), can induce toxicity from the incomplete removal of free CTAB from the CTAB-Au NPs.\(^ {26,27}\) Au NPs can also cause cells to undergo potentially damaging morphological changes. The actin fibers of human dermal fibroblasts (HDF) cells had been shown to be disrupted when incubated with citrate capped Au NPs.\(^ {28}\) The cellular uptake via endocytosis of Au NPs resulted in the enlargement of lysosomes and reduced the lysosome degradation capacity by increasing its pH.\(^ {29}\) Au NPs can also alter cellular migration, and depending on the cell type can either slow down or speed up migration.\(^ {16}\)

To be able to analyze and study NP-cell interactions more reproducibly, it is thus crucial to have methods to 1) avoid the aggregation of NPs in cell media, and 2) understand both the acute and sub-lethal toxicity of NPs on cells. The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which proposed that the stability of a suspension is determined by the interaction of two opposing electrostatic and van der Waals interactions\(^ {30-32}\), suggests that aggregation of NPs can be prevented by electrostatic and/or steric repulsive forces. It thus implies that by carefully controlling the pH, ionic strength and protein concentration, it is possible to prevent NP aggregation. In order to develop a more global picture of \textit{in vitro} AuNP toxicity, the impact of NPs on acute cellular mortality should be supplemented by studies of how cell morphology, metabolism, and gene expression change following NP exposure.

The protein corona formation on Au NPs can be embraced and used as an additional electrostatic/steric stabilizer to prevent the aggregation of Au NPs in cell media. Previously, Kah
et al. showed that the protein corona can be exploited to trap DNA or drugs for triggered release, and the sequential addition of first serum protein in PBS followed by the payload resulted in smaller Au NP clusters than when added simultaneously. In this paper, we show that by incubating Au NPs sequentially first with serum proteins in nanopure deionized water, and then dispersing the protein-Au NP complexes in buffer (containing amino acids, salts and nutrients) to make up the complete cell media, aggregation can be prevented. The cellular uptake of Au NPs as well as their toxicity was shown to be significantly different depending if Au NPs are aggregated or not. We also show that the use of other biomolecules such as phospholipids to overcoat Au NPs can prevent their aggregation in cell media. Cell viability assays were supplemented with fluorescent confocal images which showed that while cells are still viable, cell morphology is changed. Overall, our results highlight that the aggregation of Au NPs in cell media can be prevented, and should be factored in as a major component of NP-cell interaction in in vitro uptake and toxicity assays.

5.2. Materials and Methods

5.2.1. Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium citrate tribasic dihydrate (Na₃Ct·2H₂O, ≥99%), poly(allylamine hydrochloride) (PAH, M.W. 15 000 g/mol) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, ≥99.5%) were obtained from Sigma-Aldrich and were used as received. 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC) and L-α-phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole) (ammonium salt) (F-lipid) in chloroform were obtained from Avanti Polar Lipids and were used as received. Fetal bovine serum (lot #: A45D03D) was obtained from Gemini Bio-products. Fluorescein phalloidin and
DAPI was obtained from Invitrogen and used as suggested. Coomassie (Bradford) protein assay was obtained from Thermo Scientific. Ultrapure deionized water (17.9 MΩ, Barnstead Nanopure II) was used for all solution preparations, which was further sterile filtered as needed. A solution of HEPES buffer (20 mM, pH 7) was prepared in ultrapure deionized water. Glassware was cleaned with aqua regia and rinsed thoroughly before use.

5.2.2. Synthesis and Coating of Gold Nanoparticles

Au NPs (diameter 20 nm) were synthesized by the boiling citrate method as previously described with modifications. Briefly, 2.5 mL of 0.01 M HAuCl₄·3H₂O was added to 97.5 mL of ultrapure water in a 200 mL Erlenmeyer flask and heated to a gentle boil with stirring. An aqueous solution of sodium citrate (5% w/w, 2 mL) was added and the solution was allowed to boil for another 30 min. During this period, the color of the solution changed slowly to a deep red. Another portion of sodium citrate (5% w/w, 0.2 mL) was added and the solution was allowed to boil for another 20 min, following which the solution was allowed to cool slowly to room temperature while stirring. The hydrodynamic diameter measured by dynamic light scattering was 32.3 ± 0.2 nm.

Poly(allylamine hydrochloride) (PAH) Au NPs were made by first purifying 1 mL of citrate Au NPs by centrifugation. The Au pellet was collected and resuspended in 1 mL of water. To this purified Au NPs pellet, 100 μL of NaCl (0.1 M) and 200 μL of PAH (10 mg/mL) were added simultaneously, and the solution vortexed for 10 s. The NPs were allowed to incubate overnight or longer before being purified by centrifugation.
5.2.3. Lipid Coating of Gold Nanoparticles

A 1:1 weight ratio of POPS:LPC was used to coat Au NPs. F-lipid was doped at 10% of the total lipid mass. Briefly, a total of 1 mg of lipid (0.5 mg of each POPS and LPC) in chloroform was dried under a stream of nitrogen. The lipid film was then further dried under vacuum for about 6 h, after which 2 mL HEPES buffer was added to give a final concentration of 0.5 mg/mL. The mixture was sonicated for about 60 min to totally suspend the lipids, affording a clear colorless solution. To make lipid coated PAH (L-PAH) Au NPs, purified PAH Au NPs was added directly to the lipid mixture and mixed (1 pmole PAH Au NPs in 0.5 mg lipids). The mixture was allowed to incubate overnight before purification by centrifugation at 600 rcf for 30 min. The supernatant was again centrifuged at 3000 rcf for 20 min. For hybrid lipid coated (HL) Au NPs, purified citrate-capped NPs was first added to lipid mixture (1 pmole citrate Au NPs in 0.5 mg lipids), followed by 2 µL C_{18}SH (0.5 mg/mL ethanol). The mixture was mixed and incubated overnight at room temperature, then centrifuged at 4000 rcf for 25 min.

5.2.4. Cell Media

Cell media for growth of HDF cells and Au NP studies was made in house. Regular cell media was made from high glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate and supplemented with 10% FBS and 1% pen-strep. For the two steps addition of cell media, serum free 2x high glucose DMEM was supplemented with 8 mM L-glutamine, 2 mM sodium pyruvate and 3.0 g/L sodium bicarbonate. FBS was diluted to 20% with sterile water and when needed, added to 2x DMEM in a 1:1 ratio.
5.2.5. **Incubation of Gold Nanoparticles in Cell Media**

Au NPs were added to cell media in 2 ways. Au NPs were centrifuged, purified, pelleted and the concentration measured. For Au NPs added directly to cell media, an aliquot of Au NPs was taken and added to a 15 mL tube. Cell media was then added to the tube and pipetted up and down several times to disperse the Au NPs. For sequential addition of Au NPs to cell media, an aliquot of Au NPs was first added to a 15 mL tube, followed by the addition of 20% FBS and mixed. An equal volume of buffer (with other amino acids, salts and nutrients) was then added to the Au NP solution and mixed thoroughly by pipetting.

5.2.6. **Bradford Assay**

Au NPs, after incubation with serum proteins, were centrifuged 3 times with 1x phosphate buffer saline (PBS) to remove excess unbound proteins. The Au NP pellet was incubated with 5 µL KCN (1 mM) until all the Au NP had dissolved. The protein sample was then made up to 150 µL in 1x PBS. Samples (150 µL) were first added to 96 well plates, followed by 150 µL of Bradford assay reagent and mixed well. The mixture was allowed to incubate for about 10 min before analyzing the absorbance at 590 nm using a plate reader. Standard curves were made using albumin standards in 1x PBS from 0–200 µg/mL.

5.2.7. **Staining of HDF Cells for Confocal Imaging**

HDF cells were plated at a density of 20,000 cells/dish in 35 mm MatTek dishes and allowed to grow overnight. Au NPs (1 nM) were added and the cells incubated again overnight. After incubation, HDF cells were washed 2 times with 1x PBS and fixed with 4% paraformaldehyde (in 1x PBS) for 30 min. HDF cells were then washed 3 times with 1x PBS.
with 5 min incubation each. The cells were permeabilized with 0.5% Triton X-100 for 10 min and washed 3 times with 1x PBS for 5 min each. HDF cells were then incubated with 10% goat serum (in 1x PBS, 0.1% NaN₃) for 1 h before washing again 3 times with 1x PBS. Fluorescein phalloidin was added and HDF cells incubated for 30 min, following a 3 times wash with 1x PBS. DAPI was then added to the cells for 10 min and washed 3 times with 1x PBS before imaging under confocal microscope.

5.2.8. Instrumentation

A microcentrifuge (Eppendorf model 5418, Fisher-Thermo Electron) was used in various steps of synthesis and purification as detailed above. Standard absorption spectra were taken on a Cary 500 Scan UV-vis-NIR spectrophotometer at a scan rate of 30 nm/sec. Dynamic light scattering and zeta potential measurements were performed on a Brookhaven Zeta PALS instrument. Absorbance from 96 well plates was measured at 590 nm on a SpectraMax absorbance plate reader. Fluorescence confocal images were taken on a Zeiss LSM 710.

5.3. Results

5.3.1. Addition of Au NPs to Cell Media

Four types of 20 nm spherical Au NPs were investigated: anionic citrate-capped Au NPs, cationic poly(allylamine hydrochloride) (PAH) coated Au NPs, and two types of lipid coated Au NPs (Scheme 5.1). A 1:1 lipid mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (anionic, POPS)/1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (zwitterionic, LPC) was used to coat Au NPs. These lipids, which we have used before, were conjugated onto Au NPs in two ways.³⁵ By utilizing the hydrophobic interactions of the acyl tails with octadecanethiol
Scheme 5.1. Types of Au NPs Used and Surface Ligands.\textsuperscript{a}

\begin{itemize}
  \item Citrate Au NP
  \item PAH Au NP
  \item L-PAH Au NP
  \item HL Au NP
\end{itemize}

\textsuperscript{a}Note: ligands are not drawn to scale. PAH coating is shown as a blue layer on top of the citrate Au NPs. Subsequent AuNP lipid coatings are shown in a cutaway view.
(C\textsubscript{18}SH)-functionalized Au NPs, a hybrid lipid layer on Au NPs with the inner leaflet comprising of C\textsubscript{18}SH and the outer leaflet comprising of lipids can be made (HL Au NPs). Alternatively, by using electrostatic attractions between the polar headgroup of lipids with positively-charged PAH Au NPs, lipids can be coated onto PAH Au NPs (L-PAH Au NPs). UV-vis spectra of these Au NPs in water showed that after lipid coating, red shifts in the plasmon peak maxima of ~1-3 nm was seen, signifying a change in the refractive index around the Au NP after functionalization (dotted spectra, Figure 5.1a, b). Dynamic light scattering (DLS) data showed an increase in the hydrodynamic diameter after lipid functionalization: ~6 nm for HL Au NPs and a larger increase of ~128 nm for L-PAH Au NPs, possibly suggesting multilayered lipid formation on PAH Au NPs (Table 5.1). Upon lipid coating, the zeta potential became more negative (-51.9 ± 1.3 mV for HL Au NP and -48.7 ± 1.3 mV for L-PAH Au NP), reflecting the presence of POPS (negatively charged lipid) in the lipids on Au NPs.

The four types of Au NPs were incubated with cell media in the following two ways: 1) direct incubation with the full cell media (10% fetal bovine serum (FBS) in buffer with amino acids, salts and nutrients), or 2) sequential addition by first incubating Au NPs with 20% FBS in water, followed by the addition of 2x buffer (containing amino acids, salts and nutrients) in a 1:1 ratio (Scheme 5.2). As compared to incubation with just bovine serum albumin (BSA) alone, these methods mimic the complexity of the environment. When Au NPs were incubated directly in cell media, aggregation was observed for citrate and PAH Au NPs (Figure 5.1). The UV-vis plasmon band red shifted and broadened, signifying the presence of larger aggregates as compared to their respective Au NP suspensions in water. Visually, a red to purple color transition of the Au NP suspension was observed. However, when first incubated with 20% FBS, and subsequently added to an equal volume of 2x buffer, no aggregation was observed even after
Figure 5.1. UV vis spectra of 20 nm Au NPs from a) the direct addition to cell media and b) the sequential addition first to 20% FBS followed by 2x buffer. Black = citrate Au NPs; red = PAH Au NPs; blue = L-PAH Au NPs; green = HL Au NPs. The corresponding Au NPs in water is shown as the dotted spectra. Inserts: Photographs showing Au NP color after addition to bio-fluids.
Table 5.1. Hydrodynamic Diameter from Dynamic Light Scattering (DLS), \( \zeta \)-Potential Changes and Protein Adsorbed per 20 nm Au NP (protein/NP) When in Water, When Added Directly to Cell Media and When Added Sequentially to Cell Media.

<table>
<thead>
<tr>
<th>Au NP Surface</th>
<th>Water</th>
<th>Direct Addition</th>
<th>Sequential Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLS (nm) ( \zeta )-potential (mV)</td>
<td>DLS (nm) ( \zeta )-potential (mV)</td>
<td>protein/NP&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>citrate</td>
<td>32.3 ± 0.2 -19.2 ± 1.2</td>
<td>83.2 ± 1.1 -22.7 ± 0.4</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>PAH</td>
<td>34.7 ± 0.3 +16.6 ± 1.6</td>
<td>169.1 ± 7.2 -18.8 ± 0.6</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>L-PAH</td>
<td>163.2 ± 1.6 -48.7 ± 1.3</td>
<td>150.2 ± 1.2 -27.4 ± 0.8</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>HL</td>
<td>38.4 ± 0.3 -51.9 ± 1.3</td>
<td>43.1 ± 2.0 -10.8 ± 2.2</td>
<td>36 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured using Bradford assay with bovine serum albumin as calibration.
Scheme 5.2. Addition of Au NPs to Cell Media via Direct or Sequential Methods.$^a$

FBS = fetal bovine serum.
an overnight incubation. In this case, no peak broadening was observed in the UV-vis spectra and the Au NP suspensions remained red in color. Lipid coated Au NPs (HL and L-PAH) were stable in both methods, suggesting that the lipid layer can effectively reduce Au NP aggregation.

DLS data showed increase in hydrodynamic diameter on going from water, to direct or sequential addition of cell media for most Au NPs (Table 5.1). An increase in the hydrodynamic diameter of citrate Au NPs was seen when directly added to cell media (from 32.3 ± 0.2 nm to 83.2 ± 1.1 nm), but when sequentially added to cell media, only a small increase was observed (from 32.3 ± 0.2 nm to 36.8 ± 0.3 nm). The diameter of PAH Au NPs increased from 34.7 ± 0.3 nm in water to 169.1 ± 7.2 nm in direct addition to cell media, but only to 66.8 ± 1.1 nm in the sequential addition method. HL Au NPs maintained their hydrodynamic diameters in all 3 types of solutions, at 38.4 ± 0.3 nm in water, 43.1 ± 2.0 nm in direct addition to cell media and 35.8 ± 0.4 nm in sequential addition. Interestingly, sequential addition of L-PAH Au NPs to cell media showed a larger hydrodynamic size increase of 315.0 ± 5.7 nm than when directly added to cell media (150.2 ± 1.2 nm).

The amount of protein adsorbed from the media per Au NP was quantified using the Bradford assay (Table 5.1). With the exception of L-PAH Au NPs, when sequentially added to cell media, citrate, PAH and HL Au NPs had similar amounts of protein adsorbed on the 20 nm Au NPs (47 ± 3, 45 ± 1 and 44 ± 1 protein/NP respectively). Approximately twice as much protein was adsorbed onto L-PAH Au NPs (85 ± 2 protein/NP), probably due to the larger size after multilayered adsorption of lipids onto PAH Au NPs. When Au NPs were incubated directly in cell media, more variation was observed. The amount of protein adsorbed per Au NP for citrate, PAH, L-PAH and HL Au NPs was 22 ± 1, 43 ± 4, 69 ± 4 and 36 ± 2 respectively.
5.3.2. Interaction of Au NPs with HDF Cells

Au NPs from both addition methods were incubated with human dermal fibroblast (HDF) cells to study the impact of surface chemistry and sequence of addition on cell viability. Au NPs were incubated with HDF cells for at least 24 h and the relative cell viability was quantified (Figure 5.2a, b). When Au NPs were added directly in cell media, incubation of HDF cells with citrate, L-PAH and HL Au NPs did not result in significant decrease of cell viability at up to 1 nM Au NP concentration. In the presence of PAH Au NPs, the relative cell viability decreased to close to 0 % at the 1 nM PAH Au NP concentration. In comparison, when Au NPs were sequentially added to cell media, relative cell viability remained high at about 100 % for citrate and HL Au NPs. These Au NP types did not significantly reduce HDF cells cell viability at up to 4 nM Au NP concentration. With L-PAH Au NPs, a slight decrease in relative cell viability to (78 ± 27) % was observed at 4 nM Au NP. HDF cells incubated with PAH Au NPs after sequential addition to cell media had a relative cell viability of (102 ± 4) % at 1 nM PAH Au NP concentration; HDF cells relative cell viability only decreases to (12 ± 4) % at 4 nM PAH Au NP concentration, four-fold the concentration as compared to PAH Au NPs in cell media.

The uptake of Au NP by HDF cells was quantified using ICP-MS (Figure 5.2c). Au NP uptake by HDF cells was largest for Au NPs that had been directly added to cell media. 2.3–4.8 x 10^5 Au NPs were detected per HDF cell when incubated with citrate, PAH or L-PAH Au NPs. With HL Au NPs, (3.2 ± 2.5) x 10^4 Au NP were found per HDF cell. In contrast, sequential addition of Au NPs to cell media resulted in a smaller Au NP uptake by HDF cells. With citrate Au NPs, a significant decrease in uptake was observed: only (250 ± 130) Au NP/cell was detected, a thousand-fold decrease. Uptake of PAH, L-PAH and HL Au NPs by HDF cells were decreased but less drastically; PAH and L-PAH Au NPs uptake was decreased by half to about
Figure 5.2. Cell viability assay of HDF cells in a) direct addition to cell media and b) sequential addition to cell media. Citrate Au NPs (black), L-PAH Au NPs (blue) and HL Au NPs (green) do not cause significant decrease in HDF cell viability in both conditions. PAH Au NPs (red) cause significant decrease in cell viability at 1 nM when directly added to cell media, but only at 4 nM when sequentially added. c) ICP-MS results quantifying Au NP uptake per HDF cell in direct addition (black) or sequential addition to cell media (red). Au NP concentration used is 0.1 nM.
(1.2–2.3) \times 10^5 \text{ Au NP/cell}, while with HL Au NPs, a 20-fold decrease was observed ((1.6 \pm 0.3) \times 10^3 \text{ Au NP/cell}).

While Au NPs are non-toxic to HDF cells at low concentrations, cell morphology has been shown to be influenced by the uptake of Au NPs.\textsuperscript{28} In particular, actin fibers were observed to be disrupted when incubated with citrate Au NPs.\textsuperscript{28} To further explore the effect of Au NPs on cells, HDF cells incubated with Au NPs (1 nM) using both addition methods were stained for F-actin fibers using fluorescent phalloidin and imaged using fluorescence confocal microscopy. HDF cells incubated with Au NPs using the direct method is shown in Figure 5.3. Control HDF cells (without Au NPs) had well defined F-actin fibers. Incubation of HDF cells with citrate Au NPs disrupted the F-actin fibers, as judged by many globular fluorescent dots within the cell. In comparison, F-actin fibers were still prominent when incubated with HL Au NPs, although some fluorescent dots can be seen, signifying that some disruption still occurred. At 1 nM PAH Au NP concentration, HDF cells were not viable: all observed HDF cells were shrunk, lost their multipolar nature and the F-actin fibers were not clearly defined. At the same concentration, L-PAH Au NPs did not totally disrupt F-actin fibers of HDF cells; while the fibers can still be seen, they were not as well defined as control cells. In contrast, incubation of HDF cells with Au NPs in using the sequential method at the same concentration disrupted F-actin fibers of HDF cells to a lesser extent for all types of Au NPs (Figure 5.4).

The high scattering cross section of Au NPs allows them to be easily located under the reflectance mode of confocal microscopy. Large scattering was observed from HDF cells when incubated with citrate and L-PAH Au NPs, and to a lesser extent HL Au NPs, suggesting large uptake of Au NPs by HDF cells (Figure 5.3). In contrast, much less scattering was observed for HDF cells incubated with citrate Au NPs with sequential addition to cell media (Figure 5.4a).
**Figure 5.3.** Representative confocal images of control HDF cells and HDF cells exposed to citrate, PAH, L-PAH and HL Au NPs (1 nM) directly added to cell media for 24 h. Actin was stained with fluorescein phalloidin, nucleus was stained with DAPI and Au NPs was detected using reflectance mode at 633 nm.
Figure 5.4. Representative confocal images of HDF cells exposed to citrate, PAH, L-PAH and HL Au NPs (1 nM) sequentially added to cell media for 24 h. Actin was stained with fluorescein phalloidin, nucleus was stained with DAPI and Au NPs was detected using reflectance mode at 633 nm.
HDF cells incubated with PAH and L-PAH Au NPs showed large scattering from Au NPs, reflecting the large uptake of Au NPs.

A critical question regarding intracellular Au NPs is if the surface chemistry is changing on entering the cell. To understand if the lipids are displaced from Au NPs when they are uptaken into HDF cells, the lipid layer on HL and L-PAH Au NPs were doped with a fluorescent lipid (F-lipid) and incubated with HDF cells. In the absence of Au NPs, fluorescence from the F-lipid was not detected, signifying that uptake of lipid vesicles alone by HDF cells is not favorable (Figure 5.5). With L-PAH Au NPs carrying F-lipid, fluorescent vesicles (~0.8 µm) could be detected inside HDF cells. In addition, a weaker fluorescence was observed to be diffused all over the cell membrane, even after multiple washing steps. F-lipid on any of the Au NPs did not produce any fluorescence, as judged by confocal microscopy. Therefore the observation of F-lipid in HDF cells suggests that PAH Au NPs aid in the uptake of biomolecules into cells and this layer can be stripped from Au NPs after cellular entry with a 16 h incubation. Fluorescence from F-lipid was not observed when HDF cells were incubated with HL Au NPs. This can be due to either the lipids remained trapped on the Au NP surface or the fluorescence is too weak to be detected due to the lower uptake of HL Au NPs.

5.4. Discussion

A previous study have shown that the protein corona formation on Au NPs is dependent on the type of cell culture media and time, and that the nanoparticle-protein interaction can be mediated by the different components of cell media. In our investigations, we have further broken down the cell media into its buffer and FBS components, such that their respective impact on Au NPs can be studied.
Figure 5.5. Representative confocal images of control HDF cells and HDF cells exposed to HL and L-PAH Au NPs (1 nM) doped with fluorescent lipids. Nucleus was stained with DAPI and Au NPs was detected using reflectance mode at 633 nm. Control cells were treated with F-lipids only.
The initial surface chemistry of the AuNP strongly influences its tendency to aggregate in cell media. When citrate and PAH Au NPs were added to cell media using the sequential method, no significant aggregation of Au NPs was observed. The addition of FBS alone to Au NPs allowed for the complete formation of a protein corona, which potentially stabilizes Au NPs to the harsh conditions of ionic strength changes introduced by the salts added subsequently. On the other hand, while the protein corona can still form on citrate and PAH Au NPs when introduced directly to cell media, the concurrent, and possibly faster kinetics of aggregation of Au NPs due to the salts prevents complete passivation of Au NP surface before aggregation occurs. This suggests that aggregation of Au NPs in cell media is due to the exposure of Au NPs to salts prior to the formation of a well-developed protein corona. Multilayers of lipids on L-PAH Au NPs (but not HL Au NPs) also seem to influence protein binding and aggregation; DLS data showed larger increase from sequential addition compared to direct addition.

It was observed that lipid coated Au NPs (HL and L-PAH Au NPs) did not aggregate when introduced directly into a high-salt cell media. These results suggest that biomolecules such as proteins and lipids can be used to mitigate Au NP aggregation. Based on the DLVO theory, the attractive van der Waals interaction must be counter balanced by a potential barrier introduced by repulsive electrostatic interactions from the electric double layer of NPs for them to be well dispersed in solution.\textsuperscript{30–32} For NPs, this potential barrier is small and can be easily overcome by a change in the ionic strength of solution. However, unlike Au NPs, due to the shape and the non-uniform distribution of charges in proteins, the electrostatic interactions between proteins can have both attractive and repulsive interactions and cannot be fully described by DLVO theory.\textsuperscript{37} In a high salt medium such as cell media, proteins were shown to
stabilize NPs through solvation forces.\textsuperscript{38} It is this energy penalty that increases the intermolecular repulsion and hence stabilizes protein and lipid coated Au NPs from aggregation.\textsuperscript{39}

Under our experimental conditions, fewer proteins adsorb onto citrate Au NPs when they are aggregated compared to when they are well-dispersed, while similar amounts of proteins adsorb onto PAH Au NPs regardless of their aggregation state. PAH Au NPs had 43 ± 4 protein/NP from direct addition to cell media (aggregated) and 45 ± 1 protein/NP from sequential addition to cell media (not aggregated), while citrate Au NPs in direct addition (aggregated) had 22 ± 1 protein/NP and 47 ± 3 in sequential addition (not aggregated). At equilibrium, the size and surface curvature of NPs may influence the extent of protein corona formation. Given that the total surface area exposed is smaller for aggregated Au NPs than for non-aggregated Au NPs, fewer proteins would be able to adsorb on aggregated Au NPs.

Interestingly, lipids did not reduce protein corona formation on Au NPs. In fact, even more proteins bound onto L-PAH Au NPs than the other types of Au NPs. It is possible that lipids can trap lipophilic proteins in the media. This has been shown for lipid coated Au NRs, where lipophilic analytes can be trapped on the Au NR surface for enhanced detection.\textsuperscript{40}

In our study, we limited the sedimentation of Au NPs by using smaller 20 nm Au NPs. Previously, we have shown that larger 90 nm Au NPs as well as Au NRs, because of their mass, can sediment in a relatively short amount of time (~3.5 h for 90 nm Au NPs and ~14.1 h for Au NRs to fall 1 mm).\textsuperscript{16} Similarly, using Stokes’ law, the settling velocity for 20 nm Au NPs (assuming the Au NPs remain well-dispersed) is $3.9 \times 10^{-3}$ nm/s, which translates to $\sim 7.1 \times 10^4$ h or $\sim 3000$ days to fall a distance of 1 mm. Such a time frame is much longer than a typical Au NP-cell incubation study (~1-2 days), and the effects from sedimentation should be negligible.
Indeed, for the duration of our experiments (~1-2 days), non-aggregated Au NPs were observed to remain in suspension.

When aggregated (direct addition) citrate and PAH Au NP were added to HDF cells, more Au NP were taken up by cells. This is not surprising as aggregated Au NPs are denser and settle to the bottom of the plate much faster (where the cells are). The sedimented Au NPs, either on the plate surface or on HDF cells, increased the local Au NP concentration at the substrate interface, resulting in a larger cellular uptake. However, by ensuring that the Au NPs are well dispersed, a much lower Au NP uptake was observed. In fact, for well-dispersed Au NPs, completely different trends in the relative Au NP uptake were observed. In the absence of aggregation, the uptake of the citrate Au NPs was almost negligible compared to the other AuNPs tested. The toxicity of PAH Au NPs was also found to be dramatically altered when using either aggregated (direct addition) or non-aggregated (sequential addition) samples. However, the aggregation of citrate Au NP did not affect the relative cell viability of HDF cells. Similar results have been observed in other studies and are found to be dependent on various factors such as cell type and NP surface: transferrin-coated Au NPs (aggregated) are non-toxic to HeLa, A549 cells and MDA-MB-435 cells\textsuperscript{14} while single walled carbon nanotubes were shown to be toxic to mice only when aggregated.\textsuperscript{41} The toxicity of cationic NPs is possibly due to strong interactions with cell membranes, and resulted in the disruption of membrane integrity.\textsuperscript{42} In our work, the aggregation of cationic PAH Au NPs increased its concentration at the bottom of the dish (where the cells are), and hence possibly enhanced its toxicity. Given that proteins adsorbed onto Au NPs, it is highly likely that the underlying coating layers on the Au NPs can influence cellular response, and also implies that the protein corona is evolving as it enters the cell.
A total of \((4.8 \pm 0.3) \times 10^5\) PAH Au NP/cell was found in HDF cells using the direct addition method (aggregated PAH Au NPs, 0.1 nM), while when added sequentially (non-aggregated PAH Au NPs, 0.1 nM), \((2.3 \pm 0.4) \times 10^5\) PAH Au NP/cell was seen. At the same dose, the uptake of PAH Au NPs was twice as much when aggregated as compared to non-aggregated. The relative cell viability of HDF cells in well dispersed PAH Au NPs only decreases to \(\sim 10\%\) at 4 nM, while in aggregated PAH Au NPs the relative cell viability decreases to \(\sim 0\%\) at 1 nM, indicating that the effective toxic dosage of PAH Au NPs is four-fold higher when non-aggregated. While these results suggest that aggregated PAH Au NPs are uptaken at a higher rate and are more toxic, it is not possible to deduce correlation from causation; the uptake of PAH Au NPs may not be linearly related to PAH Au NP concentration and toxicity can be due to either an increased uptake of PAH Au NPs or the disruptive process of PAH Au NP size and accumulation in HDF cells. In addition, since a fraction of ICP-MS results may be due to Au NPs on the cell surfaces that are not washed away (in addition to internalized Au NPs), this fraction might be larger with aggregated PAH Au NPs, and hence cannot be correlated to toxicity.

Confocal imaging showed that F-actin fibers were disrupted to a various extents depending on Au NP type. Compared to control HDF cells, incubation of all Au NPs types to HDF cells resulted in varying decrease in F-actin fiber intensity and thickness, with the appearance of actin dots. Similar to results reported by Pernodet et al., incubation of citrate Au NPs to HDF cells showed F-actin fiber disruption.\(^{28}\) The F-actin disruption was more obvious and pronounced with aggregated (direct addition) citrate and PAH Au NPs and suggest either F-actin de-polymerization or a lack of F-actin polymerization and formation. Since HDF cells are exposed to a higher relative concentration of Au NPs when aggregated, it suggests that the F-actin disruption is Au NP concentration dependent. Indeed, when non-aggregated citrate and
PAH Au NPs are used, F-actin disruption occurred to a lesser extent. The incubation of lipid Au NPs with HDF cells helped reduced but did not eliminate F-actin fibers disruption, as judged by the appearance of actin dots. Since phalloidin stains specifically for F-actin, the actin dots observed are unlikely to be due to G-actin (monomer) accumulation. Increasing evidence showed that in addition to its cytoskeletal role, actin also plays an essential role in the endocytotic process.\textsuperscript{43,44} It is thus possible that the presence of actin dots in HDF cells is due to an increase in endocytotic events that occur at the cell surface due to Au NP uptake. Our interpretation of actin dots formation is in line with previous results, which showed that actin dots only form at the ‘top’ surface of HDF cells.\textsuperscript{28}

To investigate the lability of surface ligands on Au NPs, lipid coated Au NPs were doped with F-lipid and incubated with HDF cells. It was observed that only when exposed to L-PAH Au NPs did HDF cells show fluorescence due to F-lipid. Since the lipid vesicles (POPS/LPC/F-lipid) was shown to not be actively taken up by HDF cells, it can be concluded that the uptake and presence of F-lipid was aided by the endocytosis of L-PAH Au NPs. The bright fluorescence from F-lipid in HDF cells is believed to be due to 1) L-PAH Au NPs uptaken in extremely large quantities and 2) the multilayered lipid adsorption allowed for more F-lipid to be adsorbed per PAH Au NP. While bright fluorescent spots can be detected on HDF cells, probably due to endocytosized L-PAH Au NPs, fluorescence can also be detected throughout the cell surface. The delocalization of F-lipid from endocytotic vesicles implies that surface ligands on PAH Au NPs can be stripped from the surface. It also further implies that the underlying surface chemistry is equally important in facilitating the dissociation of the lipid layer, i.e. the electrostatic interaction between the lipid layer and PAH layer can be disrupted upon entering the cell. In addition, coupled with the relative cell viability of HDF cells when incubated with L-
PAH Au NPs, the data further showed that the toxicity of the underlying layer cannot be discounted.

5.5. Conclusion

In conclusion, we have demonstrated how the formation of the protein corona can be used as a method to prevent Au NP aggregation in cell media. We show that the surface chemistry of Au NPs and its sequence of addition to cell media can elicit differential cellular response. Even before endocytosis into HDF cells, the surface chemistry affects Au NP aggregation in cell media. However, by first adding Au NPs to FBS and then subsequently to the buffer, aggregation can be avoided. Aggregation of Au NPs can also be avoided by over-coating Au NPs with other biomolecules such as lipids. The aggregation state of the AuNPs is shown to strongly influence both cellular toxicity and Au NP uptake: non-aggregated PAH Au NPs are four-fold less toxic to HDF cells than aggregated PAH Au NPs and the uptake of non-aggregated citrate Au NPs is a thousand-fold less than aggregated citrate Au NPs. The aggregation state of the AuNPs can also influence the sub-lethal toxicity of AuNPs in vitro, specifically their effect on cell morphology. Upon uptake of Au NPs, the F-actin formation is disrupted and actin dots are predominant, possibly a response to the increase in endocytotic processes. The actin fiber disruption is more pronounced when aggregated AuNPs enter the cells. The lipid layer on L-PAH Au NPs was also seen to dissociate from PAH Au NP on entering the cell. In light of our findings, we suggest that future experiments regarding NP-cell interaction should be carefully regulated for NP aggregation and that the literature should be perused with this in mind.
5.6. Acknowledgements

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5.7. References


CHAPTER 6

Nanovacuums: Nanoparticle Uptake and Differential
Cellular Migration on a Carpet of Nanoparticles

6.1. Introduction

Gold nanoparticles (Au NPs) are excellent model NP systems for biological studies because of their low cytotoxicity and large absorption and scattering coefficients. These properties have enabled biomedical applications of Au NPs in areas such as chemical sensing, imaging, drug delivery and targeting. While the promise of nano/biotechnology is great, the understanding of nanoparticle mechanisms of interaction with biological entities has only been in recent years probed and questioned. For example, gold nanospheres (Au NSs) are preferentially taken up by HeLa cells as compared to gold nanorods of similar sizes. Au NSs of 40–50 nm diameter showed the largest uptake by human breast cancer cells as compared to other sizes. A net negative surface charge on the Au NPs was also shown to be preferred for Au NP uptake into human keratinocyte cells. Albanese and Chan showed that the degree of Au NP aggregation, a common but unavoidable occurrence when Au NPs are added to cell media, can influence cellular uptake. Recently, a study showed that the inherent density of Au NPs created a concentration gradient within the cell chamber, and cells at the bottom of the chamber were exposed to larger amounts of Au NPs as compared to the top. Most work on Au NPs has

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focused on their cytotoxicity. However, the changes in cellular behavior in the presence of Au NPs is potentially more interesting. For example, the study of cell migration is an area of active research as it has implications in wound healing, remodeling, and cancer metastasis. Cells undergoing repeated polarized extension-contraction cycles, which when coupled with adhesion and de-adhesion to the substrate, causes cell migration. In particular, for single cell migration, a 5 step process has been identified: (1) leading edge formation, (2) adhesion and traction force generation, (3) focalized proteolysis, (4) actomyosin contraction, and (5) rear-end retraction. This model is active in both normal and neoplastic single cell migration, the rate of which is influenced by the cell type and cellular environment. As long ago as 1977, Au NPs with diameters of 200–400 nm were used to visualize cell migration through the formation of tracks under darkfield illumination. This assay was used to track 3T3 fibroblast cells and human keratinocytes after fixation. However, because the Au NPs were plated on the substrate before the cells, cell adhesion to the substrate could be compromised. More recently, it had been shown that the uptake of Au NPs by human dermal fibroblast (HDF) cells can influence the formation of intracellular actin fibers, thus can indirectly affect cell adhesion and spreading. Taken together, previous work suggests that with improvements in Au NP size, shape, and surface control, the time is ripe to study cellular function such as cell migration with well-defined nanostructures.

Here, we study the effect of sedimented Au NPs on cell migration. In a typical setup, prostate carcinoma (PC3) or HDF cells were first plated to about 60–70% confluency. The cells were plated such that cell–cell interactions were minimized and cells were allowed to freely diffuse. The Au NPs then added to the cell media were allowed to sediment slowly based on their intrinsic density onto the glass substrate, and the cellular behavior was observed over a period of
at least 8 h (Scheme 6.1). The media was left intact during the experiments. We show that cells in vitro, using their leading edge, can “clean” a surface deposited with gold nanospheres (Au NSs) and long gold nanorods (Au NRs). The measured mean square displacement (MSD) and mean cumulative square distance (MCSD) values of the cell show that the “vacuuming” of Au NPs by cells is deeply intertwined with cellular migration and may relate to the known differences in migration mechanisms of PC3 and HDF cells.

6.2. Materials and Methods

6.2.1. Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium citrate tribasic dehydrate (Na₃Ct·2H₂O, ≥99%), hydroquinone (≥99%), sodium borohydride (>98%) hexadecyltrimethylammonium bromide (CTAB, >99%), poly(allylamine hydrochloride), (PAH, M.W. ~15,000), poly(acrylic acid, sodium salt) solution (PAA, M.W. ~15,000, 35 wt. % in H₂O), poly(sodium 4-styrenesulfonate) (PSS, M.W. ~70,000), poly(diallyldimethylammonium chloride) solution (PDADMAC, M.W. <100,000, 35 wt. % in H₂O), filipin III (≥85%) and chlorpromazine hydrochloride were obtained from Sigma Aldrich and were used as received. Ascorbic acid (>99%) was obtained from Acros Organics. Phosphate buffer saline without Ca²⁺ and Mg²⁺ (PBS, 10X) was obtained from Lonza and diluted to 1X before use. Methoxy(polyethylene glycol) thiol (mPEG, M.W. 5,000) was obtained from Nanocs. Calcein AM, ethidium homodimer-1, Hoechst 33342 and Cellmask Orange plasma membrane stain were obtained from Invitrogen and used as per staining protocol. Polydimethylsiloxane (PDMS, Sylgard 184) was obtained from Dow Corning. Prostate carcinoma (PC3) and human dermal fibroblast (HDF) cells were obtained from ATCC. All cell media were prepared in house (PC3
Scheme 6.1. Experimental Setup and Uptake of Au NPs

“(a) Schematics of experimental setup. Cells were first plated at low densities. Au NPs added were allowed to sediment (1−2 h) and cell migration observed. (b) Cartoon of the uptake of Au NPs on a substrate by a generic cell. The cell probes the surface for Au NPs. As the cell migrates, Au NPs are taken up via the leading edge and subsequently endocytosized at the cell body.”
medium: Ham's F-12K (kaighns modification, Sigma N3520) with 1.5g/L sodium bicarbonate supplemented with 10% FBS and 1% pen-strep. HDF medium: high glucose DMEM with 1mM sodium pyruvate (Mediatech 50003) and 3.7g/L sodium bicarbonate supplemented with 10% FBS and 1% pen-strep). Ultrapure deionized water with 17.9 M\(_2\) (Barnstead Nanopure II) was used for all solution preparations. CTAB solution was made to 0.1 M in deionized water. PAH, PAA, PSS and PDADMAC solutions were made to 10 mg/mL in 1 mM NaCl solution. mPEG solution was made to 1mM in deionized water. Glassware were cleaned with aqua regia and rinsed thoroughly before use.

### 6.2.2. Synthesis of Gold Nanoparticles

Gold nanospheres (Au NSs) (diameter 90 nm) were synthesized using 12 nm Au NPs as seeds and hydroquinone as the reducing agent.\(^{22}\) Seed Au NPs (diameter 12 nm) were synthesized using the boiling citrate method but with 3 mL of 1% sodium citrate. HAuCl\(_4\)·3H\(_2\)O solution (1% w/v, 2 mL) was centrifuged at 17,000 rcf for 1 hr after which the top 1 mL was obtained and added to 97.25 mL ultrapure deionized water and 0.75 mL of the 12 nm NP solution. The solution was then stirred rapidly at room temperature and 0.22 mL of 1% w/w sodium citrate was added, followed immediately by 1 mL of 0.03 M hydroquinone. The solution was allowed to stir overnight. Au NSs produced by this method yielded a diameter of 89 ± 13 nm under TEM. The hydrodynamic diameter measured using dynamic light scattering was 90.8 ± 0.3 nm.

Long gold nanorods (Au NRs) were synthesized using the three step synthesis, starting from gold seeds.\(^ {23}\) The seeds were made by adding HAuCl\(_4\)·3H\(_2\)O solution (0.01 M, 0.25 mL) to CTAB solution (0.1 M, 9.75 mL). Ice cold NaBH\(_4\) solution (0.01 M, 0.6 mL) was added and the mixture stirred vigorously for 10 min. The brown solution was allowed to sit for about 1 h before
use. Two ependorff tubes (labeled A and B) containing H\textsubscript{A}uCl\textsubscript{4} solution (0.01 M, 0.25 mL) and CTAB solution (0.1 M, 8.75 mL) and a flask (labeled C) containing H\textsubscript{A}uCl\textsubscript{4} solution (0.01 M, 2.5 mL) and CTAB solution (0.1 M, 90 mL) were made up. Ascorbic acid (0.1 M, 0.05 mL in A and B, 0.5 mL in C) was added and mixed until the solutions turned colorless. Seed solution (1 mL) was added to A and mixed. After 15 sec, 1 mL of A was added to B and mixed. After 30 sec, the entire solution of B was added to C and mixed. The solution was stored overnight for the gold nanorods to fully grow before purification. Au NRs made by this method had an aspect ratio of 14 (294 ± 112 nm by 21.1 ± 3.6 nm).

6.2.3. Polymer Coating on Gold Nanoparticles

Au NSs (0.01 nM, 1 mL) were purified once and Au NRs (0.1 nM, 1 mL) were purified twice via centrifugation. The gold pellet was resuspended in 1 mL deionized water, following which NaCl solution (10 mM, 0.1 mL) and polyelectrolyte solution (10 mg/mL, 0.2 mL) was added simultaneously. The solution was vortexed and allowed to incubate overnight before purification via centrifugation. Au NSs and NRs were at least triple coated in this study (Au NS: PAH/PAA/PAH (positive) or PAH/PAA/PAH/PAA (negative); Au NR: PSS/PDADMAC/PSS (negative) or PSS/PDADMAC/PSS/PDADMAC (positive)). For mPEG coating on Au NPs, mPEG (1 mM, 10 \( \mu \)L each addition) was added to the purified Au NPs in a three step addition with a 1 h interval.

6.2.4. Fabrication of Cell Chamber

The cell chamber is a simple setup made of a glass slide, PDMS and a cover slip. A 10 mm diameter hole was first drilled into the glass slide. PDMS of 18 mm by 18 mm by 1 mm and
18 mm by 18 mm by 9 mm were made and a hole with a 10 mm diameter punched out. It is crucial to wash the PDMS multiple times at this point to remove excess unreacted monomers and initiator. This was performed by continuously stirring the PDMS in the following solvent: pentane for 24 h, pentane 24 h, xylene 2 h, xylene 24 h, xylene 24 h, 200 proof EtOH 2 h, EtOH 24 h, EtOH 12 h, and DI water 24 h. The PDMS was then dried overnight in an oven at 70 °C. The glass slide and cover slip (thickness = 1.5) were cleaned in piranha solution (3:1 H₂SO₄:30% H₂O₂) for 1 h, washed thoroughly with DI water and dried under a stream of nitrogen. (Caution: Piranha solution is extremely reactive with organic matter.) To assemble the cell chamber, the glass slide and PDMS (18 x 18 x 1 mm) was first placed in an UV ozone cleaner for 3 mins and both exposed sides placed in contact. The PDMS side of the glass slide was again place face up in the UV ozone cleaner with a cover slip for 3 mins and both exposed side placed in contact. The cell chamber was then placed in an oven at 70 °C overnight to facilitate bonding of PDMS to glass. This gives a cell chamber with a thickness of 2 mm (glass slide thickness plus PDMS). The cell chamber was pre-coated with poly-lysine (0.3 mL, 1mg/mL) for 2 h before the cells were plated. To increase the volume of the cell chamber, the thicker PDMS (18 x 18 x 9 mm) can be placed in contact with the glass slide. The total volume of media that can be added to this setup is 1 mL. Before imaging, the top PDMS was removed and the glass slide covered with another cover slip on top to reduce evaporation.

### 6.2.5. Incubation of Cells with Au NPs

PC3 cells (plating density 80,000) and HDF cells (plating density 20,000) were plated and allowed to adhere to the surface overnight. Au NPs (0.005 or 0.02 nM) in media was added and further incubated as required. The cell chambers were always sealed with a cover slip prior
to imaging. For experiments with filipin (2.5 μg/mL) and chlorpromazine (5 μg/mL), cells were treated with the compounds and incubated for 15 mins at 37 °C before washing with 1x PBS. Au NPs in media was then added.

6.2.6. Cell Viability Assays

A Live/Dead stain kit was used to visualize the cells under fluorescence microscopy. Following washing with PBS, 0.2 mL of the stain (2.5 μL calcein AM and 8 μL ethidium homodimer-1 in 10 mL PBS) was added and allowed to incubate for 45 min before imaging. Live cells will fluoresce green while dead cells will give a red fluorescence.

6.2.7. Instrumentation

A microcentrifuge (Eppendorf model 5418, Fisher-Thermo Electron) was used in various steps of synthesis and purification as detailed above. Standard absorption spectra were taken on a Cary 500 Scan UV-vis-NIR spectrophotometer at a scan rate of 30 nm/sec. Zeta potential and dynamic light scattering measurements were performed on a Brookhaven Zeta PALS instrument. All spectra were corrected with HEPES buffer as baseline. Darkfield imaging was performed on a Zeiss Observer.Z1 microscope at 10.0 V. Time lapse images were taken using multidimensional acquisition in the Axiovision software, at every 5 mins for at least 8 h. Imaging at higher magnification was performed at every 10 s, for at least 40 mins.
6.3. Results and Discussion

6.3.1. Gold Nanoparticles and their Surface Modifications

In order to probe the sedimentation effect of nanoparticles on cells, large and hence heavier nanoparticles were specifically chosen. Two types of Au NPs were used, Au NSs and Au NRs. (Note: The abbreviation Au NPs will be used to describe both types of nanoparticles.) Au NSs with a diameter of 90.8 ± 0.3 nm and Au NRs with aspect ratio of ~14 (294 ± 112 nm by 21.1 ± 3.6 nm) were synthesized as previously published.\textsuperscript{22,23} The rate of sedimentation, or settling velocity, for the spherical 90 nm Au NS can be estimated by equating the frictional and buoyancy force to the gravitational force (Stokes’ law)\textsuperscript{25}

$$v_s = \frac{2(\rho_p - \rho)}{9g} \eta \frac{R^2}{g}$$

where $v_s$ is the Au NSs’ settling velocity (m/s), $\rho_p$ is the Au NS density (19 320 kg/m$^3$), $\rho$ is the water density (1000 kg/m$^3$), $\eta$ is the viscosity of water (1.002 mPa·s), $g$ is the gravitational acceleration (9.8 m/s$^2$), and $R$ is the radius of the Au NS (45 × 10$^{-9}$ m). Au NSs of 90 nm have a settling velocity of 81 nm/s. For Au NRs, due to their anisotropic shape, the translational frictional force of each Au NR depends on its orientation in solution.\textsuperscript{25} However, because the Au NRs are randomly dispersed in solution, it can be assumed that all orientations are present at any given point of time and the average frictional coefficient can be used. This can be obtained from the friction ratio (cylinders/spheres)

$$f_t = \frac{\mu}{6\pi\eta R_e}$$

where $\mu$ is the translational friction coefficient for Au NRs and $R_e$ is the equivalent radii of a sphere with equal volume.\textsuperscript{26} For the case of Au NRs with aspect ratio 14,

$$R_e = \left(\frac{3}{2p^2}\right)^{\frac{1}{3}} \left(\frac{1}{2}\right)$$
where \( p = \frac{L}{2b} \), \( L \) being the long axis of the nanorod and \( b \) being the radii, which gives \( R_e \) as 28.97 nm. Assuming the Au NRs to be cylinders, \( f_i \) is given as

\[
 f_i = \frac{3/2 \pi^2}{\ln p + \gamma} 
\]

\[
 \gamma = 0.312 + \frac{0.565}{p} + \frac{0.100}{p^2} 
\]

For Au NRs of aspect ratio 14, the \( f_i \) obtained is 1.696, giving \( \mu \) to be \( 9.28 \times 10^{-10} \) Pa·s. The buoyancy force is the volume of solvent that is displaced by the Au NR and the gravitational force is the weight of the Au NR. By equating the upward frictional and buoyancy force to the downward force due to gravity, \( v_s \) for Au NRs can be estimated

\[
 v_s = \frac{\pi^2 h g (\rho_p - \rho)}{\mu} 
\]

where \( r \) is the radius and \( h \) is the length of the Au NR (assumed cylindrical). The value \( v_s \) for Au NR is 20 nm/s. This translates to \( \sim 3.5 \) h for 90 nm Au NPs and \( \sim 14.1 \) h for 300 nm \( \times \) 20 nm Au NRs to fall a distance of 1 mm, half the height of the cell chamber, demonstrating that both Au NSs and Au NRs have a high tendency to sediment over time. However, in our experiments Au NP aggregation in cell media increases the rate of sedimentation such that the cells’ “vacuuming effect” can be observed after \( \sim 1\sim 2 \) h.

The surface chemistry of Au NPs was modified using layer-by-layer polyelectrolyte deposition.\(^{27}\) Au NSs had initial surface ligands comprising of citrate and hydroquinone, imparting an overall negative charge. By incubating positively charged poly(allylamine hydrochloride) (PAH, MW \( \sim 15 \) 000 g/mol) followed by negatively charged poly(acrylic acid) (PAA, MW \( \sim 15 \) 000 g/mol), the \( \zeta \)-potential of the Au NSs can be varied (Table 6.1). For Au NRs, since they are synthesized with the positively charged surfactant, cetyltrimethylammonium bromide (CTAB), a positively charged polyelectrolyte, poly- (diallyldimethylammonium bromide)
Table 6.1. Nanoparticle ζ-Potential in Water, Cell Diffusion Coefficients, Cell Velocities, and Apparent Au NP Cellular Uptake Per Minute for PC3 and HDF Cells When Incubated with Au NPs\(^a\)

<table>
<thead>
<tr>
<th>Au NP coating</th>
<th>ζ-potential (mV)</th>
<th>PC3</th>
<th>HDF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diffusion coefficient (µm(^2)/min)</td>
<td>cell velocity (µm/min)</td>
<td>apparent uptake rate (NP/min)</td>
</tr>
<tr>
<td>Au PAH</td>
<td>12.8 ± 0.2</td>
<td>0.61 ± 0.04</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>Au PAA</td>
<td>32.3 ± 0.7</td>
<td>10.5 ± 0.2</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>Au mPEG</td>
<td>-31.9 ± 1.7</td>
<td>4.2 ± 0.1</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>Au PDADM AC</td>
<td>-24.8 ± 0.9</td>
<td>3.2 ± 0.1</td>
<td>0.40 ± 0.15</td>
</tr>
<tr>
<td>Au NR PSS</td>
<td>39.1 ± 3.0</td>
<td>6.6 ± 0.1</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>Au NR mPEG</td>
<td>-18.6 ± 2.6</td>
<td>10.6 ± 0.4</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>3.5 ± 3.0</td>
<td>7.9 ± 0.1</td>
<td>0.33 ± 0.03</td>
</tr>
</tbody>
</table>

\(^a\)Cell diffusion coefficients (mean ± standard error of mean) are obtained from MSD plots against time (MSD)(t) = 4Dt. Cell velocities (mean ± standard error of mean) are measured from plots of distance travelled against time. Apparent Au NP uptake was estimated from cell velocities at [Au NP] = 0.02 nM. (Au NS, gold nanospheres; Au NR, long gold nanorods).
chloride) (PDADMAC, MW < 100 000 g/mol) was first adsorbed, followed by negatively charged poly(sodium 4-styrenesulfonate) (PSS, MW ~ 70 000 g/mol). Au NPs used in this study were at least triple wrapped with polyelectrolyte coatings. A minimum of three layers of polyelectrolyte coating on the nanoparticles was found to eliminate the intrinsic cytotoxicity of CTAB. For a neutral coating, Au NSs and Au NRs were coated with methoxy- (polyethylene glycol) thiol (mPEG, MW ~ 5000 g/mol). The Au NPs were then purified extensively to remove excess ligands.

With the exception of mPEG Au NSs, the ζ-potential of the Au NPs reflects the final polymer used to wrap the Au NPs (Table 6.1). It is possible that the initial surface ligands (citrate, hydroquinone, and their byproducts) are trapped in the mPEG layer, conferring a net resultant nonzero ζ-potential.

6.3.2. Uptake of Sedimented Gold Nanoparticles by PC3 and HDF Cells

To study the influence of sedimented Au NPs on cells, a house-made cell chamber was constructed using readily available materials: glass slide, coverslip, and polydimethylsiloxane (PDMS). This gave us the flexibility of maintaining a small volume of media over the cells (~ 160 mm³) and thus achieving higher nanoparticle concentrations, as well as the ability to image the cells with an inverted darkfield microscope. Cells were plated on the bottom of the polylysine-coated coverslip (PC3 cells at 80 000 cells/cm²; HDF cells at 20 000 cells/cm²), were allowed to adhere, and then grow to about 60–70% confluent before imaging. At this cell density, the cells are still relatively far apart from each other such that their movement is still only inherently random diffusion, thus simplifying our analysis for the uptake-influenced migration (vide infra). Once the cells were plated and grown to the desired confluency, cell
media spiked with gold nanoparticles was used to replace the growth media. The cells were then left to incubate in the presence of gold nanoparticles without further external disturbance; the media was not changed during the course of the imaging. (The small volume of the media made it difficult to change media without disturbing the cells.)

Darkfield images of PC3 and HDF cells incubated with different Au NSs and Au NRs were taken at 5 min intervals for at least 8 h (movies 1, 2). In addition, differential interference contrast (DIC) imaging (63× magnification) was performed at 10 s intervals for about 40 min, allowing for small changes at the cells’ leading edge to be discerned with greater certainty (movie 3). Under darkfield imaging, Au NPs appear as bright yellow-orange spots due to their inherent large scattering ability. These Au NSs and Au NRs were observed to be stationary over long periods of time (before cell interaction), indicating that they have sedimented on the glass surface. In some cases, aggregation caused the Au NSs and Au NRs to sediment as clumps. Regardless of surface coating, Au NP size and shape, cell migration was observed to vacuum Au NPs from the surface, leaving a “cleared” trail of Au NPs in its wake (Figure 6.1). Au NPs were vacuumed by the cells at their leading edge, and subsequently transported toward the cell body. This phagokinetic cell migration is random in nature and does not seem to preferentially favor regions of Au NPs. DIC imaging showed that the filopodia (cytoplasmic projections) and lamellipodia (thin, sheetlike membrane protrusions) at the leading edges of cells were constantly probing the surface. Interaction of the filopodia and lamellipodia with Au NPs caused the Au NPs to be lifted off the substrate onto the cell surface. Over time, the cells became brighter and brighter under darkfield imaging, while the nucleus remained dark, suggesting high levels of Au NP internalization (Figure 6.2).

† All movies of cell movement in the presence of Au NPs are available online at: http://pubs.acs.org/doi/suppl/10.1021/nl400972r.
Figure 6.1. Selected darkfield images (10× magnification) of the interaction of PC3 cells with sedimented PDADMAC Au NRs (0.02 nM). Images are taken at a 5 min interval over at least 8 h. The trail of one cell is outlined with white dashed lines.
Figure 6.2. Uptake of PAH Au NSs into HDF cells. (a) Darkfield image of HDF cells immediately after the addition of PAH Au NSs, and (b) darkfield image of HDF cells after 24 h. The nuclei remained dark while the cell bodies appeared highly scattering due to internalization of PAH Au NSs.
Live-dead staining of the cells after the 8 h incubation show that the cells are still alive, proving that the cells are still viable after sweeping up the Au NPs (Figure 6.3, 6.4). TEM images also show that large numbers of Au NPs were clustered in apparent endosomes, suggesting that all the Au NPs are taken into the cells by endocytosis (Figure 6.5, 6.6). The Au NP uptake by cells was estimated using the cleared tracks from darkfield imaging (Table 6.1). It is assumed that all the Au NPs sediment and are homogenously dispersed on the substrate and the cells. However, this will overestimate uptake, since some fraction of the nanoparticles are also expected to stay suspended in the media. In addition, the evolving nature of nanoparticle surface charge and aggregation state makes it difficult to measure sedimented versus suspended nanoparticles without stopping the experiment. Since we do not observe the dark tracks that result from cellular uptake of nanoparticles being filled in over time with new nanoparticles, we do not think that further sedimentation of nanoparticles is occurring within the detection limits of the microscope. The cell migration observed is thus a global response due to a combination of effects from the sedimented and suspended gold nanoparticles. While it is likely that some gold nanoparticles in suspension will be taken up, we believe that the change in migration due to suspended gold nanoparticles is minimal compared to the sedimented gold nanoparticles. We are currently in the process of improving our experimental setup so as to isolate these two effects.

The detection limit of Au NPs with darkfield microscopy was estimated from observations of scattering from known Au NP concentrations and determined to be about 120 Au NP/μm² (0.001 nM). At this concentration, if only Au NPs on the substrate will be vacuumed up, an estimate of 300 000–500 000 Au NPs are estimated to be taken up by cells after 8 h. At Au NP concentration of 0.02 nM, HDF cells incubated with PAH Au NSs exhibit the highest uptake rate at 23 100 ± 4900 Au NP/min, while when incubated with mPEG Au NRs, the lowest rate of
Figure 6.3. Live dead staining of HDF cells after at least 8 h incubation with a) PAH Au NSs, b) PAA Au NSs, c) mPEG Au NSs, d) PDADMAC Au NRs, e) PSS Au NRs and f) mPEG Au NRs. From left to right: fluorescence staining for dead cells (ethidium homodimer-1, red fluorescence), fluorescence staining for live cells (calcein AM, green fluorescence), dark field imaging of corresponding cells and combined images.
Figure 6.4. Live dead staining of PC3 cells after at least 8 h incubation with a) PAH Au NSs, b) PAA Au NSs, c) mPEG Au NSs, d) PDADMAC Au NRs, e) PSS Au NRs and f) mPEG Au NRs. From left to right: fluorescence staining for dead cells (ethidium homodimer-1, red fluorescence), fluorescence staining for live cells (calcein AM, green fluorescence), dark field imaging of corresponding cells and combined images.
Figure 6.5. TEM images of HDF cells after an overnight incubation with a) PAH Au NSs, b) PAA Au NSs, c) mPEG Au NSs, d) PDADMAC Au NRs, e) PSS Au NRs and f) mPEG Au NRs. Micrographs show Au NSs and Au NRs localized in endosomes in all cases.
Figure 6.6. TEM images of PC3 cells after an overnight incubation with a) PAH Au NSs, b) PAA Au NSs, c) mPEG Au NSs, d) PDADMAC Au NRs, e) PSS Au NRs and f) mPEG Au NRs. Micrographs show Au NSs and Au NRs localized in endosomes in all cases.
12 900 ± 2800 Au NP/min was observed. For PC3 cells, the uptake rate ranges from 16, 500 ± 3200 Au NP/min when incubated with PAH Au NSs to 22 900 ± 4400 Au NP/min when incubated with PSS Au NRs. Over 8 h incubation, it is expected that about 6−10 million Au NPs will be taken up per cell for both PC3 and HDF cells. These values are a lot higher than the reported 2000−4000 Au NP/cell after 6−24 h incubation for HeLa, A549, MDA-MB-435 and HT-29 cells.8,11,30 This suggests that most of the Au NPs “swept up” by the cells remain on the cell surface and are not internalized. Previous studies have shown that endocytosis of Au NPs by cells can occur via a clathrin-mediated (50−200 nm) or caveolin-mediated (>500 nm) pathway.31 Therefore, in our studies, we blocked these pathways with chlorpromazine (clathrin inhibitor) and filipin (caveolin inhibitor) and the cellular uptake of Au NPs was still observed. Darkfield images showed that cell migration still resulted in Au NP being vacuumed from the surface onto the cell bodies, thus suggesting that clathrin and caveolin are not required for vacuuming (Movie 4). TEM images showed much fewer Au NPs being endocytosized, demonstrating that vacuuming and endocytosis are separate events (Figure 6.7).

These results demonstrate that Au NPs interaction with cells in vitro can be generally divided into three stages: (1) interaction at the cell edge, (2) transfer to the cell body, and (3) endocytosis. Interaction of cells and Au NPs mostly occurred via the filopodia and lamellipodia at the leading edge. As the Au NPs were not tightly bound to the substrate, the constant probing by the cells can dislodge the Au NPs, resulting in them being picked up by cells and transported toward the cell body.
Figure 6.7. TEM images of PC3 cells after treatment with filipin and chlorpromazine. PC3 cells were incubated with PDADMAC Au NRs. Micrographs show a significantly reduced amount of endocytosized Au NRs in cells.
6.3.3. Gold Nanoparticles’ Influence on Cell Migration

The presence of Au NPs on substrates can potentially assist or hinder cellular adhesion to the substrate, and hence influence cell migration. The usual method to measure cell migration is by calculating the mean square displacement (MSD) of the cells:

\[
\text{MSD}(t) = \langle [r(t+t_0)-r(t_0)]^2 \rangle = 4Dt
\]

where \( r(t_0) \) is the initial position of the cell and \( r(t + t_0) \) is the new position after time \( t \), \( \langle \cdots \rangle \) denotes a combined average overall trajectories for all cells, and \( D \) is the diffusion coefficient. If the cells are sufficiently far apart and do not interfere with each other, random motion is assumed. MSD were measured with respect to the center of each cell’s nucleus.

From eq 7, a linear relationship is expected when MSD is plotted against time. Results show that for both PC3 and HDF cells incubated with and without Au NPs, a linear dependence is only observed over 100 min intervals (Figure 6.8a,b). At longer time intervals, breaks and jumps were observed. Compared to cells incubated in the absence of Au NPs, PC3 cell migration in the presence of Au NPs are slower while for HDF cells, cell migration is faster with all Au NPs except PAA Au NSs. Cellular diffusion coefficients calculated from the entire MSD plots showed that Au NPs did have an influence on cell migration (Table 6.1). PC3 cellular diffusion coefficients decrease from 12.8 ± 0.2 \( \mu m^2/\text{min} \) in the absence of Au NPs to a range of 3.2 ± 0.1 to 10.6 ± 0.4 \( \mu m^2/\text{min} \) when incubated with mPEG Au NSs and PSS Au NRs, respectively. HDF cellular diffusion coefficients increase from 3.7 ± 0.1 to 4.4 ± 0.1 \( \mu m^2/\text{min} \) and 10.8 ± 0.3 \( \mu m^2/\text{min} \) when incubated with mPEG Au NRs and PAH Au NSs, respectively. When incubated with PAA Au NSs, the cellular diffusion coefficient decreases to 3.2 ± 0.1\( \mu m^2/\text{min} \).

To better represent cell migration over long durations, the mean cumulative square distance (MCSD) is used.
Figure 6.8. Influence of Au NPs on the migration of PC3 and HDF cells. (a,b) Plot of the mean-square displacement (MSD) of PC3 and HDF cells with and without Au NPs with respect to
time. Au NPs and Au NRs concentrations are fixed at 0.02 nM. Legends are as follows: black solid square, PC3 or HDF alone; red hollow square, PAH NPs; green hollow triangle, PAA NPs; blue hollow triangle, mPEG NPs; red solid square, PDADMAC Au NRs; green solid triangle, PSS Au NRs; blue solid triangle, mPEG Au NRs. (c,d) Plot of the mean cumulative square displacement (MCSD) of PC3 and HDF cells with and without Au NPs with respect to time. (e) Box plot showing the distribution of PC3’s MCSD at 8 h after incubation. Box represent the 25th to 75th percentile of sample with the line denoting the median. Mean of sample is shown as a solid square. Whiskers represent the lower and upper inner fence limit (1.5 interquartile range). Outliers are denoted as diamonds. Sample size: PC3, 22; PAH, 38; PAA, 41; PEG NPs, 29; PDADMAC NRs, 40; PSS NRs, 24; PEG NRs, 45. (f) Box plot showing the distribution of HDF’s MCSD at 8 h after incubation. Sample size: HDF, 21; PAH NPs, 14; PAA NPs, 15; PEG NPs, 19; PDADMAC NRs, 20; PSS NRs, 24; PEG NRs, 19.
\[ \text{MCSD}(t) = \langle \sum_{i=0}^{n} (|r(t_i + \tau) - r(t_i)|)^2 \rangle \]

where \( r(t_i + \tau) \) is the position of cell at time \( \tau \) from its previous position, \( r(t_i) \), where \( \tau = 5 \text{ min} \). The summation is taken overall available time points and \( \langle \cdots \rangle \) denotes a combined average over all trajectories. The MCSD measures the total distance moved by the cells over the time frame observed, while the slope of the MCSD plot against time gives information about its speed.

Results show that PC3 cell migration is reduced in the presence of Au NPs (Figure 6.8c), similar to its respective MSD plot, regardless of the surface charge and shape of Au NPs. The MCSD plots show a linear relationship with time, signifying that the cells are migrating at a constant speed. The distributions of the samples are compared at 8 h after incubation and plotted in a box plot (Figure 6.8e). On average, PC3 cells have reduced cell migration when incubated with Au NPs. The MCSD box plots at 8 h after incubation show that PC3 cells have a mean MCSD of 2200 \( \mu \text{m}^2 \). Compared to PC3 incubated with Au NPs, the mean MCSD obtained were 960 \( \mu \text{m}^2 \) for PAH NPs, 1400 \( \mu \text{m}^2 \) for PAA NPs, 850 \( \mu \text{m}^2 \) for mPEG NPs, 1100 \( \mu \text{m}^2 \) for PDADMAC NRs, 1100 \( \mu \text{m}^2 \) for PSS NRs, and 850 \( \mu \text{m}^2 \) for mPEG NRs. The average velocity of each cell samples are deduced from the total distance moved over the 8 h incubation period (Table 6.1). These values reflect that PC3 cells move slower in the presence of Au NPs. Interestingly, HDF cells show a different MCSD pattern when incubated with Au NPs (Figure 6.8d,f). HDF cells were observed to migrate faster in the presence of positively charged Au NPs (NSs and NRs) as well as PSS Au NRs than without Au NPs, and slower in the presence of mPEG Au NSs, mPEG Au NRs, and PAA Au NSs. The mean MCSD after 8 h incubation for HDF cells was 430\( \mu \text{m}^2 \), which increases to 970 \( \mu \text{m}^2 \) with PAH NPs, 660 \( \mu \text{m}^2 \) with PDADMAC NRs and 620 \( \mu \text{m}^2 \) with PSS NRs. The mean MCSD decreases to 320 \( \mu \text{m}^2 \), 340 \( \mu \text{m}^2 \), and 290 \( \mu \text{m}^2 \) when incubated with PAA NSs, mPEG NSs, and mPEG NRs, respectively. The cell velocity reflects the same trend: PAH
Au NSs causes the largest increase of velocity from $0.28 \pm 0.01$ to $0.40 \pm 0.03 \, \mu\text{m/min}$, followed by PDADMAC Au NRs and PSS Au NRs at $0.37 \pm 0.02$ and $0.35 \pm 0.02 \, \mu\text{m/min}$, respectively.

The effect of Au NS concentration on the migration of PC3 cells was further investigated. While PC3 cells move slower when incubated with Au NSs, decreasing the Au NS concentration from 0.02 to 0.005 nM did not significantly alter PC3 cell migration (Figure 6.9). While the mean MCSD decrease from 950 to 630 $\mu\text{m}^2$ for PAH NPs and 1400 to 1000 $\mu\text{m}^2$ for PAA NPs when concentration decreases from 0.02 to 0.005 nM, respectively, no difference was observed for the MCSD box plot between different Au NS concentrations.

The results here suggest that the surface charge and shape of Au NPs can potentially influence how cells “see” the glass substrate and ultimately influence their migration. First, the behavior of the Au NPs in cell media (with 10% fetal bovine serum) reflects that a protein corona is most likely forming around the Au NPs. Previous studies have shown that regardless of the surface coatings, Au NPs maintain a net negative $\zeta$-potential when incubated in cell media.\cite{30} While the initial net charge of the nanoparticles then might be identical, which therefore might make all the nanoparticles have similar electrostatic interactions with the polylysine-coated surface the experiments were performed on, the same cannot be said for the protein corona. Because of the difference in initial surface charge, the type, orientation, and concentration of proteins that form the corona may differ between nanoparticle sets and become the driving force that alters cell migration. It becomes interesting to consider, then, how tighter binding of the nanoparticles to the surface would influence cellular behavior. Second, the inherent cell migration mechanism may influence how the cells interact with the Au NP-coated surface. Most carcinoma cells, like PC3 cells, are phenotypically heterogeneous and maintain a roundish/elliptoid shape (10–30 $\mu\text{m}$).\cite{34} They have a migration velocity of about 0.1–20 $\mu\text{m/min}$,
Figure 6.9. Influence of Au NPs concentration on the migration of PC3 cells. (a) Plot of the mean cumulative square displacement (MCSD) of PC3 cells with and without Au NPs with respect to time. Au NPs and Au NRs concentrations are at 0.005 and 0.02 nM. Legends are as follows: black solid square, PC3 alone; blue hollow square, PAH NPs (0.005 nM); blue solid square, PAH NPs (0.02 nM); red hollow square, PAA NPs (0.005 nM); red solid square, PAA NPs (0.02 nM). (b) Box plot showing the distribution of PC3’s MCSD at 8 h after incubation. Box represent the 25th to 75th percentile of sample with the line denoting the median. Mean of sample is shown as a solid square. Whiskers represent the lower and upper inner fence limit (1.5 interquartile range). Outliers are denoted as diamonds. Sample size: PC3, 22; PAH NPs (0.005 nM), 40; PAH NPs (0.02 nM), 38; PAA NPs (0.005 nM), 28; PAA NPs (0.02 nM), 41.
and their movement is usually characterized by an amoeboid migration mechanism, which is dependent on propulsive, cytoplasmic streaming. Such movement is characterized as a crawling type migration achieved by short-lived and relatively weak interactions with the substrate, which pulls the cell forward. The presence of the loosely bound Au NPs implies that when the pseudopods extend, a firm grip on the substrate cannot be established and thus PC3 cells’ migration is reduced. On the other hand, fibroblast cells (large mesenchymal cells 50–200 μm wide) have elaborate cytoskeletal networks. They adhere strongly to substrates (via integrin receptors), project broad lamellipodia at the leading edge and use microtubule networks to regulate cell migration, resulting in slow movement with net speed of around 0.25–1 μm/min. HDF cells are thus more susceptible to variations in Au NP surface charges, size, and shape. Experiments are currently in progress to understand these differences.

6.4. Conclusion

In conclusion, we show here that Au NPs, when allowed to sediment and incubate with cells in vitro, can be taken up via the cells’ intrinsic migration mechanism. For PC3 cells, a general decrease in cell migration was observed, while for HDF cells, cell migration is dependent on the surface charge and shape of Au NPs. These results signify that the interaction of Au NPs with cells in vitro is nontrivial and depends critically on cell type. Provocatively, it may be possible that Au NPs have the potential to be used to reduce the metastasis potential of cancer cells as well as to aid in wound healing in mesenchymal cells.

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6.6. References


CHAPTER 7

Short Term Impact of Gold Nanoparticles on the Genetics of Cancerous and Non-Cancerous Cells: the Discovery Phase

7.1. Introduction

In the past two decades, interest in gold nanoparticles (Au NPs) for various biomedical applications has been on the rise, leading to an exponential increase in publications on Au NPs.\textsuperscript{1} The rapid rise to fame of Au NPs can be attributed to their excellent physical properties as well as the ease manipulation to include multiple functionalities.\textsuperscript{2–6} In addition, improved synthetic routes have allowed for better yield and control of Au NPs, increasing the range of size and types, as well as tighten the size distribution of Au NPs that can be produced.\textsuperscript{7–12} These factors have led to the exploration of Au NPs in many biological applications. However, given the widespread impact of Au NPs in nano-biotechnology, there is additional need to carefully characterize the influence of Au NPs on cells.

It has generally been recognized that for most Au NPs (>5 nm), uptake into cells occurs via various receptor mediated endocytotic (RME) processes.\textsuperscript{13} RME of Au NPs by cells has been shown to depend on Au NP size, shape and aggregation state, with 50 nm Au NPs being the ideal size for efficient uptake.\textsuperscript{14,15} When uptaken, untargeted Au NPs are found to reside in organelles such as endosomes, of which 75% are transported to lysosomes within 45 min of incubation.\textsuperscript{13,16}

The surface chemistry of NPs can influence its uptake into cells. PEG coated Au NPs can reduce Au NP uptake into cells,\textsuperscript{17} while transferrin coated Au NPs limits uptake of Au NPs into
cells via only transferrin receptors.\textsuperscript{15,18} The surface charge influences NP’s affinity for cell membranes, with positively charged NPs being taken up at a greater efficiency than negatively charged Au NPs.\textsuperscript{19} However, more often than not, it is the serum proteins in protein corona of Au NPs that interacts with the receptors and determines the uptake.\textsuperscript{18,20,21} This can be avoided by coating Au NPs with mPEG-alkyl-thiol, which greatly reduced both protein adsorption and cellular uptake.\textsuperscript{22}

Morphologically, changes in cell structures have been seen upon incubation with NPs. Disruption of actin fibers and tubulin cytoskeleton had been observed in cells after Au NP uptake.\textsuperscript{23,24} The uptake of Au NPs also increases the number of autophagosomes and the enlargement of lysosomes.\textsuperscript{25} Au NPs had been found to selectively accumulate in the mitochondria of A549 cancer cells.\textsuperscript{16} Macroscopically, NPs could influence cellular response due to the change in cellular environment. For example, cell mobility had been shown to be altered when cells were incubated with Au NPs, with the extend of change dependent on cell type as well as Au NP type.\textsuperscript{26} The adhesion of MC3T3-osteoblast cells to integrin-Au NP covered surface was influenced by the spacing between the Au NPs.\textsuperscript{27} In three-dimensional collagen constructs, Au NPs altered the polymerization and mechanical properties of this extracellular matrix which cumulatively influenced cardiac fibroblasts response and behavior.\textsuperscript{28,29}

The varied responses from the incubation of NPs with cells suggest that more fundamental, molecular-based interactions exist. These interactions are further complicated due to the fact that cellular networks are all inter-linked and a seemingly irrelevant knock down of even a single protein can result with adverse effects. For example, endoplasmic reticulum stress due to Au NP uptake was linked to cellular responses such as an increase in reactive oxygen species and mitochondria damage, which can lead to cell death.\textsuperscript{30} To fully study and quantify the
changes happening in cells, a fundamental and more thorough analysis of cellular response to NPs such as probing the global gene expression changes of cells in response to NPs is necessary.

In this aspect, previous studies had shown that Au NPs’ size and shape can induce different cellular pathways related to cellular stress and toxicity.\textsuperscript{31,32} Nucleic acid functionalized Au NPs as well as citrate Au NPs did not induce significant gene expression changes in HeLa cells,\textsuperscript{33,34} while mercaptohexadecanoic acid functionalized Au NPs induced more changes than PEG coated Au NPs over the 84 genes probed in human keratinocyte cells.\textsuperscript{35} When human skin fibroblasts were incubated with carbon nanotubes and nano-onions, the gene responses were found to be dependent on NP concentration.\textsuperscript{36}

We are interested in the short-term exposure (24-48 h) of cells to Au NPs. In this paper, we investigate the gene expression changes in human dermal fibroblast (HDF) cells and prostate cancer cells (PC3) after incubation of 20 nm Au NPs with different surface coatings. We showed that the impact of Au NPs depends on its initial surface chemistry, as well as the cell type. In HDF cells, gene expression changes are minimal when incubated with citrate Au NPs and hybrid-lipid-coated (HL) Au NPs but not with poly(allylamine hydrochloride)-wrapped (PAH) Au NPs and lipid-coated poly(allylamine hydrochloride)-wrapped (L-PAH) Au NPs. PAH and L-PAH Au NPs induced changes in all phases of the cell cycle of HDF cells, with a large percentage of the expressed genes in the mitosis phase. In PC3 cells, gene expression changes are strongly influenced by the surface functionalized of Au NPs. In all, our results showed that the different gene clusters are affected by the different surface chemistries on Au NPs, which ultimately influenced the degree of change and type of cellular pathways.
7.2. Materials and Methods

7.2.1. Materials

Gold(III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium citrate tribasic dihydrate (Na₃C₆H₄O₆·2H₂O, ≥99%), 1-octadecanethiol (98%, C₁₈SH), poly(allylamine hydrochloride), (PAH, M.W. 15,000 g/mole), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma Aldrich and were used as received. 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (POPS), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC) were obtained from Avanti Polar Lipids and were used as received. Trizol (Invitrogen) and RNeasy kit (Qiagen) were used in the extraction of RNA. Ultrapure deionized water (17.9 MΩ, Barnstead NANOpure II) was used for all solution preparations. Glassware were cleaned with aqua regia and rinsed thoroughly before use.

7.2.2. Synthesis of Gold Nanoparticles

Au NPs of diameter 20 nm was synthesized via the boiling citrate method as previously described.⁴⁸,⁴⁹ Briefly, 2.5 mL of 0.01 mM HAuCl₄ solution was added to 97.5 mL ultrapure deionized water and heated to a gentle boil with stirring. The solution was allowed to boil for another 5 min before 2 mL of 5% (w/w) sodium citrate was added. The solution was boiled for another 30 min until a deep red color was obtained. Another 0.5 mL of 5% sodium citrate was added to the solution and boiled for 10 min, following which the solution was allowed to cool while stirring.
7.2.3. PAH Coating of Gold Nanoparticles

A 1 mL aliquot of as-made Au NPs was centrifuged and purified. The Au pellet was collected and resuspended in 1 mL of water. To this purified Au NPs pellet 100 μL NaCl (0.1 M) and 200 μL PAH (10 mg/mL) were added simultaneously and the solution vortexed. The NPs were allowed to incubate overnight or longer before purifying by centrifugation.

7.2.4. Preparation of POPS/LPC Lipid Vesicles

A 1:1 weight ratio of POPS/LPC was used to make hybrid-lipid-coated Au NPs. Briefly, a total of 1 mg of lipid (0.5 mg of each POPS and LPC) in chloroform was dried under a stream of nitrogen. The lipid film was then further dried in vacuum for about 6 h, after which 1 mL of 20 mM HEPES buffer was added to give a final concentration of 1 mg/mL. The mixture was sonicated for about 1 h to totally suspend the lipids, affording a clear, colorless solution. Dynamic light scattering measurements of these vesicles gave an average hydrodynamic diameter of ~90 nm.

7.2.5. Synthesis of Lipid-Coated Gold Nanoparticles

A 1 mL aliquot of Au NPs were centrifuged to remove the excess ligands from the synthesis and redispersed to 0.5 mL in 20 mM HEPES buffer. For lipid-coated PAH Au NPs (L-PAH Au NPs), 0.5 mL of the 1:1 POPS/LPC lipid solution from the above section was added to PAH Au NPs and mixed. For hybrid lipid Au NPs (HL Au NPs), 0.5 mL of the lipid solution was added to purified citrate-capped Au NPs, followed by 2 μL of C_{18}SH (0.5 mg/mL in ethanol). The mixture was incubated overnight at room temperature. The mixture was then centrifuged
and the Au pellet resuspended in HEPES buffer.

**7.2.6. Incubation of Gold Nanoparticles with Cells**

HDF and PC3 cells were plated in 6-well plates and grown to confluency. Au NPs were first suspended in cell media, and then added to cells (1 nM Au NPs for PC3, 0.1 nM for HDF). The cells were incubated with Au NPs for at least 24 h before RNA extraction.

**7.2.7. RNA Extraction**

A combined Trizol extraction, followed by RNeasy purification was used. Briefly, cells were first washed thrice with PBS, and 1 mL Trizol added. The cells were homogenized by pipetting up and down several times and transferred to an Eppendorf tube. The sample was allowed to sit for about 5 min at room temperature before adding 0.2 mL chloroform. The mixture was vortexed for 20 sec, incubated for 12 min at room temperature and centrifuged at 20,000 rcf for 20 min at 4 °C. The upper aqueous phase was extracted, taking care to avoid the organic layer. To this aqueous layer, an equal amount of ethanol was added and mixed. This sample was loaded into an RNeasy column and spun at 8,000 rcf for 30 sec. The flow through was discarded and 700 μL of buffer RW1 was added to the column. The column was spun at 8,000 rcf for 30 sec and the flow through discarded. To the column, 500 μL of buffer RPE added and spun again at 8,000 rcf for 30 sec (repeated twice). The column was spun at 20,000 rcf for 1 min to get rid of remaining buffer and transferred to a new collection tube. RNA was eluted with 30 μL DEPC-treated water at 8,000 rcf for 1 min. Collected RNA was checked for purity using a Nanodrop and Bioanalyzer and stored at -80 °C until ready for microchip array analysis.
7.2.8. Microarray Labeling and Hybridization

For each sample 200 ng of total RNA was labeled using the Agilent 2-color Low Input Quickamp Labeling kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocols. Labeled samples were hybridized to a Human 4 x 44 Agilent microarray and scanned on an Axon 4000B microarray scanner at 5 um resolution.

7.2.9. Data Processing

Microarray data pre-processing and statistical analyses were done in R (v 3.0.1) using the limma package (v 3.16.7).\textsuperscript{37,38} Median foreground and median background values from the 15 arrays were read into R and any spots that had been manually flagged (-100 values) were given a weight of zero.\textsuperscript{39} The background values were ignored because investigations showed that trying to use them to adjust for background fluorescence added more noise to the data. The individual Cy5 and Cy3 values from each array were all normalized together using the quartile method and then log2-transformed.\textsuperscript{39} Correlations between the replicate spots per probe were high and were simply averaged for each sample. The positive and negative control probes were used to assess what minimum expression level could be considered "detectable above background noise" (6 on the log2 scale) and then discarded. A mixed effects statistical model was fit on the 34,127 unique probes to estimate the mean expression level for each of the NP incubations while accounting for dye effects and the correlation due to array.\textsuperscript{40,41} Probes that did not have expression values > 6 in at least 3/30 samples were discarded. Pairwise comparisons between the nanoparticles within each cell line were pulled as contrasts from the model, along with the equivalent of a one-way ANOVA test for NPs within each cell line and the overall interaction test between cell line and...
NPs. Raw p-values were adjusted separately for each comparison using the False Discovery Rate method.42

7.2.10. Functional Clustering Analysis

Gene clusters for expressed genes were generated using DAVID (Database for Annotation, Visualization and Integrated Discovery) developed at National Cancer Institute at Frederick.43 Up- and down-regulated genes were submitted and analyzed using functional annotation clustering and functional annotation chart. The classification stringency was set at medium and kappa similarity threshold was set at 0.50. Clusters were selected based on their Fisher exact p-value as well as their relevance. Cellular pathways were mapped to KEGG database, developed by Kanehisa Laboratories.44

7.2.11. Weighted Gene Correlation Network Analysis

To computational assess the different expression patterns of PC3 cells when incubated with Au NPs, Weighted Gene Correlation Network Analysis (WGCNA) was performed on a subset of the probes for the PC3 cells.45,46 We selected 4,496 probes that had a reasonable level of statistical evidence for differential expression (PC3 one-way ANOVA FDR p-value < 0.1) and reasonable amount of changed expression (at least 1.5 FC between any 2 of the 5 groups) and performed WGCNA (v 1.27-1) using the default values of the blockwiseModules() function except for: soft thresholding power $\beta = 22$, an unsigned topological overlap matrix, a minimum module size of 20 and merging similar modules at 0.1. This resulted in 16 modules containing 1197 to 21 probes, plus the "module 0" consisting of 12 probe sets that did not fit any of the 16 patterns.
7.2.12. Instrumentation

Absorption spectra of Au NPs were taken on a Cary 500 scan UV-vis-NIR spectrophotometer while absorption spectra of RNA were taken on Nanodrop 1000. Transmission electron microscopy data were obtained with a JEOL 2100 Cryo electron microscope operating at 200 kV. Zeta potential and dynamic light scattering measurements were performed on a Brookhaven Zeta PALS instrument. RNA quality was determined using a Agilent Bioanalyzer and RNA expression was probed using Agilent Human GE 4 x 44K v2 Microarray and scanned on an Axon 4000B microarray scanner (Molecular Devices, Sunnyvale, CA) at 5 µm resolution. Differential expression analysis was carried out using GenePix 6.1 software (Molecular Devices, Sunnyvale, CA).

7.3. Results

7.3.1. Global Gene Expression Changes on Au NPs Incubation

The impact of 20 nm spherical Au NPs with four different surface coatings on two types of cells was investigated (Scheme 7.1). As-made Au NPs have citrate (anionic) on the surface. By polyelectrolyte coating with poly(allylamine hydrochloride) (PAH), the chemistry is changed to an amine-terminated surface, making the Au NP cationic. We also investigated the influence of pre-coating Au NPs with biomolecules, which are believed to help improve the biocompatibility of Au NPs. Au NPs were coated with a 1:1 mixture of lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (anionic, POPS)/1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (zwitterionic, LPC)) and were allowed to adsorb differently based on the initial surface chemistry. On PAH Au NPs, the electrostatic interactions between PAH Au NPs and negatively charged POPS/LPC vesicles was favored, thus forming lipid-coated PAH Au NPs (L-
Scheme 7.1. Potential Exposure to Au NPs and Experimental Setup.\(^a\)

(a) Cartoon of potential routes to NP exposures. NP-based therapy exposes organs (targeted sites) to high levels of NPs while environmental elements expose the skin to low levels of NPs. (b) Schematics of experimental setup. (c) Different types of Au NPs used.
PAH Au NPs). Alternatively, by functionalizing citrate Au NPs with octadecanethiol (C$_{18}$SH), the hydrophobic interactions between the alky tail chains of the lipids and the inner leaflet of C$_{18}$SH formed a hybrid lipid layer on Au NPs (HL Au NPs).

Two different cell types were studied under different conditions (Scheme 7.1). Unintentional exposure to NPs (at low dosage) would most often occur via contact with the skin. As such, human dermal fibroblast (HDF) cells were investigated as a model as they would most often be the first type of cells that are in direct contact/exposure to NPs. HDF cells were incubated with Au NPs at a low concentration of 0.1 nM. Alternatively, NPs are often used at higher concentrations in biological applications, either for imaging or therapy. Prostate cancer cells (PC3) were chosen to represent typical targeted cells and were exposed to Au NPs at 1.0 nM concentrations. In both cases, cells were exposed to Au NPs for 24–48 h, during which >95% were alive before RNA extraction.

Global gene expression analysis was performed in triplicates for both cell types with the four kinds of Au NPs and differently expressed genes were clustered in heatmaps (Figure 7.1). Incubation of HDF cells with citrate and HL Au NPs showed minimal gene expression changes; the heatmap for HDF cells with citrate and HL Au NPs showed a similar color response compared to control HDF cells. In contrast, incubation of HDF cells with PAH or L-PAH Au NPs induced an inverse gene expression response; genes that were initially preferentially expressed were now suppressed while genes that were suppressed had higher expressions. Incubation of PC3 cells with Au NPs did not follow a similar gene expression response. Instead, different types of Au NPs elicited different gene expression responses from PC3 cells. In a parallel analysis, the gene expression data was analyzed using principle component analysis.
Figure 7.1. Heatmap showing changes in global gene expression of a) HDF cells and b) PC3 cells, after exposure to citrate, HL, PAH and L-PAH Au NPs. Each row represents a RNA probed and the change in expression level is shown as red, for up-regulation, and blue, for down-regulation. All samples are performed in triplicates.
(Figure 7.2). These results showed that 1) HDF and PC3 cells are distinct cell types; 2) incubation of HDF cells with citrate or HL Au NPs induced very small changes in gene expression as compared to controls; 3) incubation of HDF cells with PAH or L-PAH Au NPs induced significant changes in gene expression compared to controls; 4) the gene expression differences of HDF cells between PAH or L-PAH Au NPs were small; and 5) incubation of PC3 cells with different Au NPs elicited very different gene expression response from PC3 cells, suggesting that PC3 cells are more ‘responsive’ to the different Au NPs than HDF cells.

Table 7.1 lists the number of genes that were differential expressed with a fold change (FC) of at least ±1.5 after treatment with Au NPs (FC less than 1.0 are given as the negative inverse). Exposure of HDF cells to either citrate or HL Au NPs resulted in only a small number of differentially expressed genes, with a total of 11 and 48 genes that are differential expressed respectively (p-value < 0.05, FC < -1.5 or FC > 1.5). Exposure of HDF cells to PAH or L-PAH Au NPs elicited a bigger gene expression response, with a total of 1272 and 1285 genes expressed differently respectively (FDR < 0.05, FC < -1.5 or FC > 1.5). When incubated with citrate, HL or PAH Au NPs, more genes are down-regulated than up-regulated, and with L-PAH Au NPs, more genes are up-regulated. Similar to HDF cells, exposure of PC3 cells to PAH or L-PAH Au NPs elicited a stronger gene expression response than when exposed to citrate or HL Au NPs: the total number of genes expressed significantly altered was 80 for citrate Au NPs, 234 for HL Au NPs, 384 for PAH Au NPs and 1548 for L-PAH Au NPs. More genes were up-regulated with citrate Au NPs but down-regulated with HL, PAH or L-PAH Au NPs. Comparison of the total genes expressed differently between cell types showed that PC3 cells induced greater gene expression changes than HDF cells except for when incubated with PAH Au NPs, where less genes were changed (384 genes in PC3 and 1272 genes in HDF). These
Figure 7.2. Principle component analysis (PCA) of HDF cells and PC3 cells in three principle component axes. Each cell sample is represented as a sphere, with control cells colored as yellow, citrate Au NP incubation as green, HL Au NP incubation as red, PAH Au NP incubation as blue and L-PAH Au NP incubation as magenta.
Table 7.1. Number of Genes in HDF and PC3 Cells Which Differentially Expressed After Exposure to Au NPs.

<table>
<thead>
<tr>
<th>Au NP</th>
<th>HDF genes</th>
<th></th>
<th></th>
<th>PC3 genes</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Down-regulated</td>
<td>Up-regulated</td>
<td>Total</td>
<td>Down-regulated</td>
<td>Up-regulated</td>
<td>Total</td>
</tr>
<tr>
<td>citrate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>25</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>HL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td>18</td>
<td>48</td>
<td>144</td>
<td>90</td>
<td>234</td>
</tr>
<tr>
<td>PAH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>694</td>
<td>578</td>
<td>1272</td>
<td>367</td>
<td>17</td>
<td>384</td>
</tr>
<tr>
<td>L-PAH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>610</td>
<td>675</td>
<td>1285</td>
<td>1147</td>
<td>401</td>
<td>1548</td>
</tr>
</tbody>
</table>

<sup>a</sup>Genes are filtered with a cut-off criteria of raw p-value < 0.05 and either a fold change (FC) < -1.5 for down-regulated genes or FC > 1.5 for up-regulated genes (FC value of less than 1 are replaced by its negative inverse).

<sup>b</sup>Genes are filtered with a cut-off criteria of false discovery rate (FDR) < 0.05 and either a FC < -1.5 for down-regulated genes or FC > 1.5 for up-regulated genes.
results suggest that PAH and L-PAH Au NPs induced a greater cellular response from both HDF and PC3 cells.

Genes expressed significantly from each cell types were compared among the different types of Au NPs (Figure 7.3). The Venn diagram showed that no differentially expressed genes are common among the different Au NP incubation. Very few genes were also similarly changed when HDF cells were incubated with either citrate or HL Au NPs. More similarities were found between gene expression changes due to PAH and L-PAH Au NPs. Out of 1272 and 1285 differently expressed genes when HDF cells was incubated with PAH and L-PAH Au NPs respectively, 916 genes are commonly changed. On incubation of Au NPs with PC3 cells, very few expressed gene similarities were found between citrate and HL Au NPs. However, 20 genes after citrate Au NP incubation and 56 genes after HL Au NP incubation were expressed similarly to L-PAH Au NPs incubation. Comparison of PAH and L-PAH Au NPs also showed that 305 genes were changed similarly. Taken together, the data suggested that incubation of Au NPs elicit different responses from different cell type, and the surface chemistry strongly influenced cellular response.

7.3.2. Gene Expression Changes in HDF cells after Au NP Incubation

To highlight the impact of Au NP incubation, genes that were most significantly changed and showed the greatest FC were tabulated in Table 7.2. Incubation of HDF cells with citrate or HL Au NPs did not produce genes with FC > 2.5 or FC < -2.5, suggesting that Au NP impact is small. HDF cells incubated with PAH or L-PAH Au NPs shared a common set of genes that were differentially expressed. In addition, most of these genes were commonly regulated in both PAH and L-PAH Au NP conditions. Genes such as keratin associated protein 2-3 (KRTAP2-3),
Figure 7.3. Venn diagram comparing the genes (up and down-regulated) that showed expression changes (FC > 1.5, FC < -1.5) for a) HDF cells and b) PC3 cells. Each Venn diagram is divided according to the type of Au NP treatment. The numbers in the diagrams represent the genes detected. The number of genes that are commonly differential expressed are shown in the overlapping regions.
Table 7.2. List of Genes With the Highest Fold Change in HDF Cells After Incubation With PAH or L-PAH Au NPs.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>PAH Au NP</th>
<th>L-PAH Au NP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>FDR&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KRTAP2-3</td>
<td>keratin associated protein 2-3</td>
<td>3.62</td>
<td>9.94 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
<td>1.99</td>
<td>2.92 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCNE2</td>
<td>cyclin E2</td>
<td>3.41</td>
<td>9.14 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>DTL</td>
<td>denticless E3 ubiquitin protein ligase homolog</td>
<td>3.34</td>
<td>4.17 x 10&lt;sup&gt;-12&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAL</td>
<td>galanin/GMAP prepropeptide</td>
<td>2.14</td>
<td>5.10 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>RRM2</td>
<td>ribonucleotide reductase M2</td>
<td>3.68</td>
<td>2.91 x 10&lt;sup&gt;-11&lt;/sup&gt;</td>
</tr>
<tr>
<td>FAM111B</td>
<td>family with sequence similarity 111, member B</td>
<td>2.72</td>
<td>1.64 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>HAS2</td>
<td>hyaluronan synthase 2</td>
<td>3.20</td>
<td>1.04 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>WFDC1</td>
<td>WAP four-disulfide core domain 1</td>
<td>3.11</td>
<td>1.65 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>RCAN2</td>
<td>regulator of calcineurin 2</td>
<td>-3.41</td>
<td>3.43 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>SLC9A9</td>
<td>solute carrier family 9, subfamily A member 9</td>
<td>-3.07</td>
<td>1.92 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>SECTM1</td>
<td>secreted and transmembrane 1</td>
<td>-3.99</td>
<td>6.07 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>C5orf4</td>
<td>chromosome 5 open reading frame 4</td>
<td>-3.25</td>
<td>5.33 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADH1A</td>
<td>alcohol dehydrogenase 1A (class I), alpha polypeptide</td>
<td>-4.16</td>
<td>4.27 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>aldo-keto reductase family 1, member C3</td>
<td>-3.41</td>
<td>1.04 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAN1C1</td>
<td>mannosidase, alpha, class 1C, member 1</td>
<td>-3.59</td>
<td>3.19 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADH1C</td>
<td>alcohol dehydrogenase 1C (class I), gamma polypeptide</td>
<td>-5.48</td>
<td>4.17 x 10&lt;sup&gt;-12&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold change value of less than 1 are replaced by its negative inverse.

<sup>b</sup> Significance is judged with false discovery rate (FDR) adjusted p-values, and is based on the total gene expression sample.
cyclin E2 (CCNE2), denticuleless E3 ubiquitin protein ligase (DTL) and ribonucleotide reductase M2 (RRM2) were up-regulated and alcohol dehydrogenase 1A and 1C (ADH1A, ADH1C), mannosidase alpha (MAN1C1) and aldo-ketoreductase family 1 (AKR1C3) were down-regulated in both cases.

To understand the significance of the differential gene expression changes, and the possible biological pathway/terms that are affected, the changed genes were analyzed using high-throughput bioinformatics analytical tool DAVID (Database for Annotation, Visualization and Integrated Discovery). Incubation of HDF cells with citrate Au NPs did not yield statistically significant gene categories, suggesting that at a 0.1 nM concentration, the impact of citrate Au NPs is minimal. When incubated with HL Au NPs, changed genes impacted gene categories such as GTPase activation and phosphorylation (Table 7.3). However, it should be noted that the statistical significance of these categories were low and the number of altered genes against all the genes in the categories was also small, suggesting that the impact of HL Au NP on HDF cells is also small. We hence focus out subsequent study of HDF cells to incubation with PAH and L-PAH Au NPs.

Incubation with PAH Au NPs induced greater gene changes in gene categories related to cell cycle, organelle lumen, cell migration, nucleotide binding, apoptosis regulation, phosphorylation, transcription and protein ubiquitination. A large number of altered gene expressions were related to cell cycle, organelle lumen, cytoskeleton and nucleotide binding. Of note, genes related to cell cycle, organelle lumen, cytoskeleton and protein ubiquitination were observed to be more up-regulated than down-regulated, while in categories of cell migration, apoptosis regulation and transcription regulation, more genes were down-regulated than up-regulated. A similar trend was seen with L-PAH Au NP incubation with HDF cells: a large
Table 7.3. Selected Gene Category After Treatment of HDF Cells With Au NPs.\(^a\)

<table>
<thead>
<tr>
<th>Gene category</th>
<th>Fisher Exact p-value(^b)</th>
<th>FDR(^c)</th>
<th>No. of genes</th>
<th>% genes down-regulated(^d)</th>
<th>% genes up-regulated(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL Au NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTPase activation</td>
<td>0.047</td>
<td></td>
<td>44</td>
<td>3</td>
<td>0.63</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>0.62</td>
<td></td>
<td>100</td>
<td>3</td>
<td>0.38</td>
</tr>
<tr>
<td>PAH Au NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell cycle</td>
<td>2.5x10(^{-36})</td>
<td>4.6x10(^{-33})</td>
<td>150</td>
<td>3.74</td>
<td>15.59</td>
</tr>
<tr>
<td>Organelle lumen</td>
<td>5.7x10(^{-12})</td>
<td>8.1x10(^{-9})</td>
<td>181</td>
<td>3.35</td>
<td>6.59</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>7.8x10(^{-5})</td>
<td>0.11</td>
<td>119</td>
<td>2.24</td>
<td>6.37</td>
</tr>
<tr>
<td>Cell migration</td>
<td>1.8x10(^{-4})</td>
<td>0.32</td>
<td>25</td>
<td>8.28</td>
<td>6.51</td>
</tr>
<tr>
<td>Nucleotide binding</td>
<td>5.6x10(^{-3})</td>
<td>8.4</td>
<td>168</td>
<td>2.98</td>
<td>4.50</td>
</tr>
<tr>
<td>Apoptosis regulation</td>
<td>2.0x10(^{-3})</td>
<td>3.6</td>
<td>47</td>
<td>4.51</td>
<td>5.58</td>
</tr>
<tr>
<td>Phosphorylation regulation</td>
<td>2.0x10(^{-3})</td>
<td>3.6</td>
<td>13</td>
<td>4.61</td>
<td>2.88</td>
</tr>
<tr>
<td>Transcription regulation</td>
<td>7.5x10(^{-3})</td>
<td>13</td>
<td>195</td>
<td>4.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Protein ubiquitination</td>
<td>0.011</td>
<td></td>
<td>18</td>
<td>4.00</td>
<td>10.00</td>
</tr>
<tr>
<td>L-PAH Au NPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell cycle</td>
<td>2.8x10(^{-38})</td>
<td>5.1x10(^{-35})</td>
<td>153</td>
<td>2.71</td>
<td>17.01</td>
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<tr>
<td>Organelle lumen</td>
<td>1.5x10(^{-10})</td>
<td>2.1x10(^{-7})</td>
<td>176</td>
<td>2.31</td>
<td>7.36</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>5.0x10(^{-6})</td>
<td>7.2x10(^{-3})</td>
<td>125</td>
<td>2.17</td>
<td>6.88</td>
</tr>
<tr>
<td>Cell migration</td>
<td>2.3x10(^{-5})</td>
<td>0.043</td>
<td>27</td>
<td>9.47</td>
<td>6.51</td>
</tr>
<tr>
<td>Phosphorylation regulation</td>
<td>9.8x10(^{-5})</td>
<td>0.18</td>
<td>52</td>
<td>4.08</td>
<td>7.08</td>
</tr>
<tr>
<td>Nucleotide binding</td>
<td>1.2x10(^{-3})</td>
<td>1.9</td>
<td>171</td>
<td>2.36</td>
<td>5.26</td>
</tr>
<tr>
<td>Apoptosis regulation</td>
<td>1.2x10(^{-3})</td>
<td>2.1</td>
<td>74</td>
<td>5.22</td>
<td>3.98</td>
</tr>
<tr>
<td>Protein ubiquitination</td>
<td>0.052</td>
<td></td>
<td>62</td>
<td>1.00</td>
<td>11.00</td>
</tr>
<tr>
<td>peroxisome</td>
<td>0.098</td>
<td></td>
<td>77</td>
<td>9.71</td>
<td>0.97</td>
</tr>
<tr>
<td>Metal ion binding</td>
<td>0.4</td>
<td></td>
<td>100</td>
<td>3.55</td>
<td>2.68</td>
</tr>
</tbody>
</table>

\(^{a}\)Gene categories were generated using DAVID program with a medium stringency threshold.

\(^{b}\)One-tail Fisher Exact p-value is used for gene-enrichment analysis. The smaller the value, the more enriched the gene category.

\(^{c}\)False discovery rate (FDR) adjusted p-value.

\(^{d}\)% genes up or down-regulation based on number of genes in category listed compared to total genes in this category with the same Gene Ontology (GO) term.
proportion of changed genes were related to cell cycle, organelle lumen, cytoskeleton and nucleotide binding, of which genes related to cell cycle, organelle lumen, cytoskeleton and protein ubiquitination were more up-regulated than down-regulated. On the other hand, genes related to cell migration, apoptosis regulation and peroxisome were more down-regulated than up-regulated.

Table 7.4 and 7.5 lists the genes that are most significantly changed after Au NP incubation in gene categories cell cycle and cytoskeleton respectively. It was observed that genes up or down-regulated with PAH Au NPs were similarly expressed with L-PAH Au NPs (FC < -1.5, FC > 1.5). CCNE2 (cyclin E2) was the most up-regulated gene in the cell cycle category (FC = 3.41 for PAH Au NPs and FC = 3.56 for L-PAH Au NPs). With down-regulated genes, HMG20B (high mobility group 20B) was the most down-regulated gene for PAH Au NP (FC = -2.88) while CDKN1C (cyclin dependent kinase inhibitor 1C) was the most down-regulated for L-PAH Au NPs (FC = -2.64). In the cytoskeleton category, KRTAP2-3 (keratin associated protein 2-3) was the most up-regulated gene (FC = 3.62 for PAH Au NPs, FC = 4.39 for L-PAH Au NPs) and PPL (periplakin) was the most down-regulated gene (FC = -2.76 for PAH Au NPs, FC = -2.88 for L-PAH Au NPs).

We further investigated the cell cycle of HDF cells as it showed the greatest change when incubated with PAH and L-PAH Au NPs. Cellular division, mitosis, can be separated into the G1, S, G2 and M phases. The gene categories of the G1/S transition, G2/M transition checkpoint and M phase are tabulated in Table 7.6. Both PAH and L-PAH Au NPs implicated gene expression changes in all three categories, suggesting that PAH and L-PAH Au NPs impact the cell cycle in all phases. However, most of the gene expressions altered were linked to the M phase, with 64 expressed genes altered when incubated with PAH Au NPs and 71 expressed
Table 7.4. Most Significantly Changed Genes of HDF Cells After Au NP Incubation That Fall Under the Gene Category of Cell Cycle.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change</th>
<th>citrate Au NP</th>
<th>HL Au NP</th>
<th>PAH Au NP</th>
<th>L-PAH Au NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNE2</td>
<td>cyclin E2</td>
<td></td>
<td>1.10</td>
<td>1.28</td>
<td>3.4</td>
<td>3.56</td>
</tr>
<tr>
<td>CDC45</td>
<td>cell division cycle 45</td>
<td></td>
<td>1.25</td>
<td>1.01</td>
<td>2.90</td>
<td>2.99</td>
</tr>
<tr>
<td>BARD1</td>
<td>BRCA1 associated RING domain 1</td>
<td></td>
<td>1.16</td>
<td>1.27</td>
<td>2.67</td>
<td>2.52</td>
</tr>
<tr>
<td>ZWINT</td>
<td>ZW10 interactor, kinetochore protein</td>
<td></td>
<td>1.23</td>
<td>1.31</td>
<td>2.65</td>
<td>2.22</td>
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<tr>
<td>RAD51</td>
<td>RAD51 homolog (S. cerevisiae)</td>
<td></td>
<td>1.09</td>
<td>1.17</td>
<td>2.44</td>
<td>2.54</td>
</tr>
<tr>
<td>UBE2C</td>
<td>ubiquitin-conjugating enzyme E2C</td>
<td></td>
<td>1.00</td>
<td>1.12</td>
<td>2.27</td>
<td>2.16</td>
</tr>
<tr>
<td>CHAF1B</td>
<td>chromatin assembly factor 1, subunit B (p60)</td>
<td></td>
<td>1.20</td>
<td>1.08</td>
<td>2.22</td>
<td>2.43</td>
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<tr>
<td>MCM3</td>
<td>minichromosome maintenance complex component 3</td>
<td></td>
<td>1.10</td>
<td>1.32</td>
<td>2.20</td>
<td>2.23</td>
</tr>
<tr>
<td>E2F2</td>
<td>E2F transcription factor 2</td>
<td></td>
<td>1.15</td>
<td>1.15</td>
<td>2.17</td>
<td>2.51</td>
</tr>
<tr>
<td>CDC25A</td>
<td>cell division cycle 25A</td>
<td></td>
<td>1.23</td>
<td>1.11</td>
<td>2.02</td>
<td>2.42</td>
</tr>
<tr>
<td>G0S2</td>
<td>G0/G1 switch 2</td>
<td></td>
<td>1.11</td>
<td>1.28</td>
<td>1.99</td>
<td>2.42</td>
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<tr>
<td>HMG20B</td>
<td>high mobility group 20B</td>
<td></td>
<td>-1.35</td>
<td>-1.24</td>
<td>-2.88</td>
<td>-1.54</td>
</tr>
<tr>
<td>MAP2K6</td>
<td>mitogen-activated protein kinase kinase 6</td>
<td></td>
<td>-1.13</td>
<td>1.11</td>
<td>-2.58</td>
<td>-2.33</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>cyclin-dependent kinase inhibitor 1C (p57, Kip2)</td>
<td></td>
<td>-1.02</td>
<td>-1.25</td>
<td>-2.41</td>
<td>-2.64</td>
</tr>
<tr>
<td>TXNIP</td>
<td>thioredoxin interacting protein</td>
<td></td>
<td>-1.22</td>
<td>-1.40</td>
<td>-2.40</td>
<td>-2.25</td>
</tr>
</tbody>
</table>
Table 7.5. Most Significantly Changed Genes of HDF Cells After Au NP Incubation That Fall Under the Gene Category of Cytoskeleton.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>citrate</th>
<th>HL</th>
<th>PAH</th>
<th>L-PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRTAP2-3</td>
<td>keratin associated protein 2-3</td>
<td>1.32</td>
<td>1.48</td>
<td>3.62</td>
<td>4.40</td>
</tr>
<tr>
<td>CDC45</td>
<td>cell division cycle 45</td>
<td>1.25</td>
<td>1.01</td>
<td>2.90</td>
<td>2.99</td>
</tr>
<tr>
<td>KRT34</td>
<td>keratin 34</td>
<td>1.26</td>
<td>1.19</td>
<td>2.80</td>
<td>2.64</td>
</tr>
<tr>
<td>TPM1</td>
<td>tropomyosin 1 (alpha)</td>
<td>-1.07</td>
<td>-1.24</td>
<td>2.73</td>
<td>2.12</td>
</tr>
<tr>
<td>TUBB6</td>
<td>tubulin, beta 6 class V</td>
<td>1.28</td>
<td>1.15</td>
<td>2.41</td>
<td>2.71</td>
</tr>
<tr>
<td>ACTG2</td>
<td>actin, gamma 2, smooth muscle, enteric</td>
<td>1.08</td>
<td>1.04</td>
<td>2.34</td>
<td>2.90</td>
</tr>
<tr>
<td>AURKA</td>
<td>aurora kinase A</td>
<td>1.09</td>
<td>1.21</td>
<td>2.22</td>
<td>2.36</td>
</tr>
<tr>
<td>KIF23</td>
<td>kinesin family member 23</td>
<td>1.07</td>
<td>1.05</td>
<td>2.22</td>
<td>2.23</td>
</tr>
<tr>
<td>MCM3</td>
<td>minichromosome maintenance complex component 3</td>
<td>1.10</td>
<td>1.32</td>
<td>2.20</td>
<td>2.23</td>
</tr>
<tr>
<td>CDCA8</td>
<td>cell division cycle associated 8</td>
<td>1.08</td>
<td>-1.18</td>
<td>2.11</td>
<td>2.36</td>
</tr>
<tr>
<td>FAM83D</td>
<td>family with sequence similarity 83, member D</td>
<td>1.13</td>
<td>-1.09</td>
<td>2.05</td>
<td>2.27</td>
</tr>
<tr>
<td>CCNE1</td>
<td>cyclin E1</td>
<td>1.14</td>
<td>1.23</td>
<td>2.02</td>
<td>2.24</td>
</tr>
<tr>
<td>PPL</td>
<td>periplakin</td>
<td>-1.08</td>
<td>1.057</td>
<td>-2.76</td>
<td>-2.88</td>
</tr>
<tr>
<td>FILIP1L</td>
<td>filamin A interacting protein 1-like</td>
<td>-1.28</td>
<td>1.08</td>
<td>-1.31</td>
<td>-2.00</td>
</tr>
<tr>
<td>MYLIP</td>
<td>myosin regulatory light chain interacting protein</td>
<td>-1.14</td>
<td>-1.14</td>
<td>-1.92</td>
<td>-1.99</td>
</tr>
<tr>
<td>RHOQ</td>
<td>ras homolog family member Q</td>
<td>-1.02</td>
<td>-1.00</td>
<td>-1.20</td>
<td>-1.91</td>
</tr>
<tr>
<td>IFFO1</td>
<td>intermediate filament family orphan 1</td>
<td>-1.03</td>
<td>-1.10</td>
<td>-1.88</td>
<td>-1.85</td>
</tr>
</tbody>
</table>
Table 7.6. Analysis of Cell Cycle Gene Category After Treatment of HDF Cells With Au NPs.

<table>
<thead>
<tr>
<th>Gene category</th>
<th>Fischer Exact p-value</th>
<th>FDR</th>
<th>No. of genes</th>
<th>% down-regulated</th>
<th>% up-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAH Au NPs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1/S transition of mitotic cell cycle</td>
<td>0.025</td>
<td>36</td>
<td>9</td>
<td>1.79</td>
<td>14.29</td>
</tr>
<tr>
<td>G2/M transition checkpoint</td>
<td>2.5x10^{-3}</td>
<td>4.4</td>
<td>6</td>
<td>6.25</td>
<td>31.25</td>
</tr>
<tr>
<td>M phase of mitotic cell cycle</td>
<td>9.8x10^{-25}</td>
<td>1.8x10^{-21}</td>
<td>64</td>
<td>2.23</td>
<td>26.34</td>
</tr>
<tr>
<td><strong>L-PAH Au NPs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1/S transition of mitotic cell cycle</td>
<td>0.063</td>
<td>69</td>
<td>8</td>
<td>1.79</td>
<td>12.50</td>
</tr>
<tr>
<td>G2/M transition checkpoint</td>
<td>2.5x10^{-3}</td>
<td>4.4</td>
<td>6</td>
<td>12.50</td>
<td>25.00</td>
</tr>
<tr>
<td>M phase of mitotic cell cycle</td>
<td>1.5x10^{-30}</td>
<td>2.6x10^{-27}</td>
<td>71</td>
<td>1.34</td>
<td>30.36</td>
</tr>
</tbody>
</table>
genes with L-PAH Au NPs. In all three categories, genes were more up-regulated than down-regulated. For incubation with PAH Au NPs, genes in the G1/S transition was 14.29% up-regulated and 1.79% down-regulated, genes in the G2/M transition checkpoint was 31.25% up-regulated and 6.25% down-regulated and genes in the M phase was 26.34% up-regulated and 2.23% down-regulated. Similarly for incubation with L-PAH Au NPs, a 12.50% (vs. .79%), 25.00% (vs. 12.50%) and 30.36% (vs. 1.34%) up-regulation (vs. down-regulation) of genes was observed in the G1/S transition, G2/M transition checkpoint and M phase respectively.

Differentially expressed genes were mapped into the cell cycle pathway (KEGG pathway database). The altered genes could be mapped to the G1, S and G2 phase of cell cycle (Figure 7.4). PAH and L-PAH Au NPs induced similar gene changes, with most of the genes commonly up-regulated by both types of Au NPs. L-PAH Au NPs induced two additional gene changes: down-regulation of CDKN1A (CIP1, cyclin-dependent kinase inhibitor 1A) and up-regulation of PLK1 (polo-like kinase 1).

7.3.3. Gene Expression Changes in PC3 cells after Au NP Incubation

Similar to HDF cells, PC3 cells incubated with citrate or HL Au NPs did not produce genes that had FC > 2.5 or FC < -2.5 and were statistically significant. However, gene expression fold changes of PC3 cells incubated with PAH or L-PAH Au NPs were much greater when compared to HDF cells (Table 7.7). More genes showed greater FC when incubated with L-PAH Au NPs, especially genes that were up-regulated. The largest up-regulated gene was chemokine ligand 1 (CXCL1), which with L-PAH Au NPs showed a FC of 10.83, but only FC of -1.03 with PAH Au NP. Similarly, other up-regulated genes such as interleukin 8 (IL8), lymphotoxin beta (LTB), chromosome 15 open reading frame 48 (C15orf48), chemokine ligand 6 (CXCL6) and
Figure 7.4. Altered genes in HDF cell cycle after incubation with PAH Au NPs (red) and L-PAH Au NPs (blue). Gene mapping was obtained using DAVID and cell cycle pathway was obtained from KEGG (ref 44).
Table 7.7. List of Genes With the Highest Fold Change in PC3 Cells After Incubation With PAH or L-PAH Au NPs.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>PAH Au NP</th>
<th></th>
<th>L-PAH Au NP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>FDR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>FDR&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
<td>-1.03</td>
<td>9.64 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10.83</td>
<td>2.04 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>1.18</td>
<td>6.31 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>6.86</td>
<td>3.27 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>LTB</td>
<td>lymphotoxin beta</td>
<td>1.03</td>
<td>9.53 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>4.13</td>
<td>3.69 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>C15orf48</td>
<td>chromosome 15 open reading frame 48</td>
<td>1.11</td>
<td>5.85 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>4.036</td>
<td>9.34 x 10&lt;sup&gt;-11&lt;/sup&gt;</td>
</tr>
<tr>
<td>CXCL6</td>
<td>chemokine (C-X-C motif) ligand 6</td>
<td>1.10</td>
<td>8.09 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>3.85</td>
<td>7.52 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>BCL2A1</td>
<td>BCL2-related protein A1</td>
<td>1.13</td>
<td>8.48 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>3.54</td>
<td>2.94 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZNF768</td>
<td>zinc finger protein 768</td>
<td>-3.52</td>
<td>1.08 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>-3.05</td>
<td>1.12 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>BAK1</td>
<td>BCL2-antagonist/killer 1</td>
<td>-2.09</td>
<td>2.48 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>-3.19</td>
<td>7.81 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>LAMB2</td>
<td>laminin, beta 2 (laminin S)</td>
<td>-2.39</td>
<td>3.97 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-3.22</td>
<td>4.07 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>DDR1</td>
<td>discoidin domain receptor tyrosine kinase 1</td>
<td>-1.88</td>
<td>3.22 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>-3.23</td>
<td>3.95 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>BAP1</td>
<td>BRCA1 associated protein-1</td>
<td>-3.28</td>
<td>1.06 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>-3.36</td>
<td>1.51 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>MIB2</td>
<td>mindbomb E3 ubiquitin protein ligase 2</td>
<td>-1.76</td>
<td>1.75 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>-3.51</td>
<td>4.74 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>EIF4G1</td>
<td>eukaryotic translation initiation factor 4</td>
<td>-3.16</td>
<td>2.21 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-3.71</td>
<td>1.43 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>MUC6</td>
<td>mucin 6, oligomeric mucus/gel-forming</td>
<td>-2.08</td>
<td>2.48 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>-3.83</td>
<td>2.87 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLEC</td>
<td>plectin</td>
<td>-3.03</td>
<td>7.82 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-3.84</td>
<td>2.82 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNK2</td>
<td>tyrosine kinase, non-receptor, 2</td>
<td>-3.33</td>
<td>2.50 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>-4.37</td>
<td>4.74 x 10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>RRBP1</td>
<td>ribosome binding protein 1</td>
<td>-3.34</td>
<td>7.15 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>-4.79</td>
<td>6.16 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
<tr>
<td>NES</td>
<td>nestin</td>
<td>-5.60</td>
<td>8.60 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>-6.62</td>
<td>4.74 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold change value of less than 1 are replaced by its negative inverse.

<sup>b</sup> Significance is judged with false discovery rate (FDR) adjusted p-values, and is based on the total gene expression sample.
BCL-related protein A1 (BCL2A1) were only significantly up-regulated with L-PAH Au NP and not with PAH Au NPs. On the other hand, down-regulated genes were commonly expressed in both PAH or L-PAH Au NPs, with nestin (NES) having the highest FC of all the down-regulated genes.

In contrast to HDF cells, the heatmap for PC3 cells showed a much more complex expression pattern across the four Au NP types. To facilitate and simplify the identification of altered cellular functions, we used a weighted gene correlation network analysis (WGCNA) to find clusters (modules) of highly correlated genes across all Au NP incubations. This resulted in a total of 16 modules detected (FDR p-value < 0.1, at least 1.5 FC), with the number of genes ranging from 19-1131 (Figure 7.5 and 7.6). In each module, the gene expression levels for all genes from each Au NP incubation was summarized into a singular value, eigengene (i.e. first principle component), which roughly correspond to the average (over-expression) of gene expression values in the Au NP incubation (Figure 7.5a). These eigengenes thus allow for a simplified method to compare gene expression differences between Au NP incubations and modules. Results showed that gene expressions were significantly very different in all modules, across all Au NP incubations when compared to control cells. The eigengene values for PAH and L-PAH Au NPs were down-regulated in modules 1, 2 and 8, and up-regulated in modules 6, 10 and 13. Compared to the control, citrate and HL Au NP incubations, which showed an opposite gene expression trend, this data suggest that these modules might be linked to the molecular effect of PAH. On the other hand, HL and L-PAH Au NP incubations up-regulated the eigengene values in modules 3 and 14, and down-regulated in modules 5, 9, 15 and 16 (compared to control, citrate Au NPs and PAH Au NPs incubation which showed opposite regulation), thus suggesting that these modules might be related to the lipid layer on Au NPs.
Figure 7.5. a) Identification of modules (co-related genes) using WGCNA and conversion into a single eigengene value for each Au NP incubation (module 1). b) Bar plots of modules (modules 2–7) after PC3 cells incubation with citrate Au NP (red), HL Au NPs (green), PAH Au NPs (dark blue) and L-PAH Au NPs (light blue). Control PC3 cells are given as black. Error bar denotes triplicates.
Figure 7.6. Bar plots of modules (modules 8–16) after PC3 cells incubation with citrate Au NP (red), HL Au NPs (green), PAH Au NPs (dark blue) and L-PAH Au NPs (light blue). Control PC3 cells are given as black. Error bar denotes triplicates.
To allocate functional significance to these modules, the genes grouped in each module were analyzed using DAVID separately. In this analysis, an EASE score of 0.01 was used, and gene category were selected for using first the highest number of genes matched, followed by the lowest p-value (most significant) (Table 7.8). At least 55% of the module genes were matched to genes in the gene category. Analysis of the module genes showed that they did not simply correspond to specific cellular organelles or processes. Instead, we were able to match the genes to transcription factor binding sites (TFBS). Several modules were mapped to the same TFBS: modules 1, 2 and 8 were mapped to AREB6 (synonym: ZEB1, zinc finger E-box binding homeobox 1), modules 3, 7 and 10 were mapped to OCT1 (organic cation transporter), modules 5 and 6 were mapped to EVI1 (ecotropic viral integration site 1) and modules 4 and 14 were mapped to RFX1 (regulatory factor X 1). MEF2 (myocyte enhancer factor 2), PAX5 (paired box 5), AP4 (activating enhancer binding protein 4), YY1 (Yin Yang 1), CDPCR3 (CCAAT displacement protein cut repeats 3) and FOXJ2 (forkhead box J2) were represented by modules 9, 11, 12, 13, 14, 15 and 16 respectively.

7.4. Discussion

Gene expression analysis allowed for the global impact of Au NPs on cells to be investigated. Our results illustrate that the surface chemistry of Au NPs influences cells differently, and the extent of influence is dependent on cell type. For HDF cells, greater gene expression changes were observed with PAH and L-PAH Au NPs when compared to control cells. However, differences between these two Au NP types were minimal, with about two-thirds of the genes commonly expressed. In contrast, fewer genes were expressed differently after incubation with citrate and HL Au NPs, of which very few are common between the two Au NP
Table 7.8. List of Modules and Their Respective Transcription Factor Binding Sites (TBSF) of PC3 Cells.

<table>
<thead>
<tr>
<th>Module No.</th>
<th>Ontology Class (TFBS)</th>
<th>Module genes</th>
<th>Class genes (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AREB6</td>
<td>1131</td>
<td>79.5</td>
<td>$1.8 \times 10^{-13}$</td>
</tr>
<tr>
<td>2</td>
<td>AREB6</td>
<td>726</td>
<td>83.7</td>
<td>$3.3 \times 10^{-14}$</td>
</tr>
<tr>
<td>3</td>
<td>OCT1</td>
<td>549</td>
<td>80.0</td>
<td>$1.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>4</td>
<td>RFX1</td>
<td>344</td>
<td>56.0</td>
<td>$4.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>5</td>
<td>EVI1</td>
<td>355</td>
<td>75.8</td>
<td>$2.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>6</td>
<td>EVI1</td>
<td>215</td>
<td>82.2</td>
<td>$1.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>7</td>
<td>OCT1</td>
<td>172</td>
<td>85.7</td>
<td>$4.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>8</td>
<td>AREB6</td>
<td>161</td>
<td>82.0</td>
<td>$8.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>9</td>
<td>MEF2</td>
<td>136</td>
<td>67.7</td>
<td>$7.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>10</td>
<td>OCT1</td>
<td>95</td>
<td>90.3</td>
<td>$1.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>11</td>
<td>PAX5</td>
<td>77</td>
<td>58.3</td>
<td>$8.1 \times 10^{-1}$</td>
</tr>
<tr>
<td>12</td>
<td>AP4</td>
<td>81</td>
<td>67.5</td>
<td>$1.3 \times 10^{-2}$</td>
</tr>
<tr>
<td>13</td>
<td>YY1</td>
<td>68</td>
<td>72.1</td>
<td>$2.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>14</td>
<td>RFX1</td>
<td>26</td>
<td>73.1</td>
<td>$3.7 \times 10^{-2}$</td>
</tr>
<tr>
<td>15</td>
<td>CDPCR3</td>
<td>19</td>
<td>63.2</td>
<td>$9.6 \times 10^{-2}$</td>
</tr>
<tr>
<td>16</td>
<td>FOXJ2</td>
<td>21</td>
<td>81.0</td>
<td>$9.6 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
types. A similar gene distribution pattern was observed with PC3 cells, where a significant portion of genes were common between PAH and L-PAH Au NPs, and minimal similarity between citrate and HL Au NPs. Such differences can be understood by taking into account the lability of the surface ligands with Au NPs. The electrostatic interaction of lipid with the underlying PAH of L-PAH Au NPs is relatively weak; lipids can dissociate from the 20 nm Au NP to form larger, more stable liposomes.47 This exposes the underlying PAH layer, which could result in the similar gene expression changes when compared to PAH Au NPs. On the other hand, the hydrophobic interaction between lipids and C_{18}SH on HL Au NPs are probably a lot stronger and its dissociation unfavorable, resulting in cells responding differently from the Au NP exposure. Interestingly, only a few genes were commonly expressed between both lipid coated Au NPs samples, suggesting that the underlying chemistry on Au NPs impact lipid layer formation and ultimately influence how the cells interact with these Au NPs.

It was previously shown that the gene expression depended on NP concentration.36 Incubation of human skin fibroblasts with carbon nanotubes at different concentrations resulted in drastically different genes being expressed, with very few genes commonly expressed.36 In our study, while cells were incubated with Au NPs at fixed concentrations (0.1 nM for HDF, 1.0 nM for PC3), the uptake of Au NP per cell depended on the Au NP surface chemistry. Comparing the uptake of Au NP per cell to gene changed, a similar relationship was observed. The uptake of HL Au NPs was significantly lower compared to PAH and L-PAH Au NPs in both HDF cells, while in PC3 cells, uptake of L-PAH Au NPs is significantly greater than PAH and HL Au NPs. The number of expressed genes changed in HDF cells when incubated with HL Au NPs was significantly lower than PAH and L-PAH Au NPs, while for PC3 cells, expressed genes changed with L-PAH Au NP incubation is a lot larger than HL and PAH Au NPs. The trend, however,
cannot be used to account for gene expression changes with citrate Au NPs in HDF and PC3 cells. Uptake of citrate Au NPs was similar to PAH Au NP uptake in HDF cells and L-PAH Au NPs in PC3 cells; expressed genes changed due to citrate Au NP incubation was the lowest compared to other Au NP types for both HDF and PC3 cells. This suggests that the genes expressed are likely to be in part influenced by the endocytotic process, and in part influenced by the chemical impact per Au NP: for citrate Au NPs, even though much more Au NP is endocytosized, the influence is small as the impact per Au NP is small.

A similar number of altered expressed genes was observed in both incubations with PAH and L-PAH Au NPs, of which at least two-thirds of the expressed genes are common between these two Au NP incubations, suggesting that many similar processes/pathways are changed through incubation with PAH or L-PAH Au NPs. This is also reflected in Table 7.2, where highly expressed genes were common between PAH and L-PAH Au NPs. It is possible to rule out the influence of the metallic Au NP core: these genes were not differentially expressed across all four Au NP incubation. Since the only similarity between these two Au NPs is the PAH layer, the data suggest that the PAH layer exert a significant change in gene expression of HDF cells.

Incubation of HDF cells with PAH and L-PAH Au NPs resulted in the up-regulation of the cell cycle in all phases. CCNE2 (cyclin E2), the most up-regulated gene, is involved in the cell cycle via the G1 to S phase transition.\textsuperscript{48} It exerts its effect through a complex formation with CDK2 (cyclin-dependent kinase), which regulates cellular processes by phosphorylating downstream proteins.\textsuperscript{49} Other genes such as CDC45 (cell division cycle 45) and CDCA5 (cell division associated 5) are similarly up-regulated, as is CHAF1B (chromatin assembly factor 1, subunit b), which implied that the rate of DNA replication had increased. In parallel, CDKN1C (cyclin-dependent kinase inhibitor 1C), TXNIP (thioredoxin interacting protein) and MAP2K6...
(mitogen-activated protein kinase kinase 6) were down-regulated, suggesting that transcriptional activities were less suppressed. The up-regulation of the HDF cell cycle, and in particular the over-expression of CCNE2, is a source of concern, especially in toxicology. Cell cycle genes had been used as profile genes for metastatic cancer, with their over-expression usually signifying its presence. In particular, CCNE2 is often used as a prognostic marker for breast and prostate cancer. Up-regulation of CCNE2, without proper control, can lead to genomic instability such as chromosomal aberrations and genetic mutations, a result of the higher frequency of transcription. The up-regulation of CCNE2 in HDF cells upon PAH and L-PAH Au NP incubation suggests a possibility of HDF’s cancer progression. At a 0.1 nM Au NP concentration, the cell viability of HDF cells is still relatively high, which suggest that mutations in genes can be propagated onto daughter cells.

Gene expression for PC3 cells showed a different pattern. Heatmap of PC3 cells after Au NP incubated showed that each incubation elicited a different gene response. While little difference were observed between citrate and HL Au NPs, similar to HDF cells, a smaller difference was also seen between PAH and L-PAH Au NPs; only 308 genes were common between these two Au NP incubation. In particular, up-regulated genes between PAH and L-PAH Au NPs were not the same. The most up-regulated genes with L-PAH Au NP incubation were only minimally changed when PC3 cells was incubated with PAH Au NPs (FC < 1.2). On the other hand, down-regulated genes were commonly expressed, suggesting that the down-regulation of genes might be due to the PAH layer, while the up-regulated genes might be related to the lipids.

CXCL1 was only up-regulated when PC3 cells was incubated with L-PAH Au NPs. CXCL1 is a CXC chemokine which mediates communication between different cells. It plays
an extensive role in inflammation, angiogenesis, tumorigenesis and wound healing. Previous studies have shown that up-regulation of CXC chemokines promoted prostate tumor malignancy in PC3 cells as well as tumorigenesis of melanoma. A more recent study suggests that over-expression of CXCL1 represses tumor establishment in TRAMP-C2 cell line and also altered cell adhesion and migration in an in vitro setting. The BCL2 proteins are important cell death regulators and serves to control the release of cytochrome c from mitochondria. The protein family consists of both pro- and anti-apoptotic proteins and work in synergy to regulate apoptosis. BCL2A1 was up-regulated in only L-PAH Au NP incubation, while its antagonist, BAK1, was down-regulated in both PAH and L-PAH Au NP incubations, suggesting that the anti-apoptotic function is preferred after PAH or L-PAH Au NP incubation. Increased expression of BCL2A1 had been associated with cancer and tumor progression, while the down-regulation of BAK1 had been linked to gastrointestinal cancers. While significance of the up-regulation of CXCL1 (FC = 10.83) and BCL2A1 (FC = 3.54) with L-PAH Au NP incubation remains to be tested, the results suggest that this up-regulation may be due to the lipid layer on L-PAH Au NPs. On the other hand, down-regulation of BAK1 might be due to the PAH layer on both PAH or L-PAH Au NPs. Taken together, the data seems to suggest that Au NP uptake influences PC3 cell proliferation.

WGCNA allowed unique properties of gene expression data to be expressed and had been used to reveal unique characteristics in gene expression data. However, in our study, the modules were not found to be simple representations of cellular functions or processes. Instead, they were mapped to TFBS with higher significance, suggesting that the Au NPs does not just impact isolated cellular functions, but had a deeper molecular impact on cells. Most crucial is the AREB6 transcription factor, which was shown to map to three modules at high significance,
influencing a total of 1638 genes. AREB6 (synonym: ZEB1, zinc finger E-box binding homeobox 1) encodes a zinc finger transcription factor and transcriptionally represses cell adhesion molecules. ZEB1 is also known to negatively regulate interleukin 2, a cytokine signaling molecule in the immune system. In human prostate cancer, ZEB1 over-expression can lead to E-cadherin down-regulation, thus promoting epithelial-to-mesenchymal transition and tumor migration and invasion. As such, ZEB1 has been suggested for use as a biomarker for metastatic prostate cancer. Incubation of PC3 cells with Au NPs showed that when PAH or L-PAH Au NPs were used, negative eigengene values were obtained, suggesting the down-regulation of genes in all three modules (modules 1, 2 and 8), and hence the possible suppression of ZEB1. In contrast, incubation with HL Au NPs up-regulated eigengene values when compared to control cells, suggesting that HL Au NPs activated ZEB1. The different cellular responses to Au NP’s surface chemistries thus indicate that Au NPs can influences cells via more fundamental, molecular interactions.

7.5. Conclusion

By making use of microarray to probe differentially expressed genes, and through data mining using readily available analysis programs, the global impact of Au NPs on cells was uncovered. Incubation of HDF cells with citrate and HL Au NPs did not result in much gene expression changes at 0.1 nM, suggesting that the impact of Au NPs at this concentration is minimal. In contrast, incubation of HDF cells with PAH and L-PAH Au NPs resulted in larger gene expression changes and the cell cycle was found to be up-regulated in all phases. Analysis of PC3 cells after incubation with Au NPs showed that the different surface chemistries of Au NPs induced different gene responses. By using network analysis (WGCNA), it was shown that
Au NPs impact PC3 cells via more fundamental, molecular interactions. Overall, our results showed that the surface chemistries on Au NPs as well as the cell type can influence cellular response, and as such needed to be specifically fine-tuned if to be used in biomedical applications.

7.6. Acknowledgements

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7.7. References


CHAPTER 8

Future Paths for Exploration

8.1. Probing Biomolecules Distribution, Orientation and Conformation on Gold Nanoparticles

The importance of the nanoparticle-biomolecule interface is only recently starting to be recognized, and there are still many unknowns about these interactions: much of this complexity is contributed by the numerous types and possible surface functionalization of nanoparticles, as well as the various kinds of interactions with about $10^5$ different proteins. The protein corona composition on nanoparticles is also actively being studied and had been shown to change significantly in terms of the amount of bound proteins over time.\(^1\) We have focused our work on simplified systems containing either one or two types of biomolecules (phospholipids and $\alpha$-synuclein), and have demonstrated that biomolecules have each their own preferred adsorption pattern which can affect their overall physiology. However even these studies have proved to be challenging and several key issues need to be addressed for more progress in this field:

1) Stellacci et al. had previously used scanning tunneling microscopy to analyze ligand separation on nanoparticle surfaces.\(^2\) As his method is intriguing and has been the subject of much debate, alternative methods are required to corroborate his findings.\(^3\) We have used staining methods followed by TEM imaging to look for patchiness on Au NPs, however this method is qualitative at best. A better alternative might be Cryo-TEM, which can be used to preserve the organic layer while imaging. Dyes that undergo J-
aggregation can be conjugated to surface ligands and the shift in fluorescence wavelength studied, to learn how ligands phase separate on surfaces (although this is difficult on Au NPs). Alternatively, ligands can be cross linked to ‘fix’ their position and analyzed using NMR.

2) Given that proteins fold according to various intra-molecular interactions such as electrostatic, hydrophobic bonding, Van der Waals forces and salt bridges, a generalized picture of nanoparticle-biomolecule interaction can be obtained by investigating the contributions of each factor at the nanoparticle interface. To this end, the biomolecular interaction at the Au NP interface can be simplified into two regions: 1) interaction between biomolecules and 2) interaction at the biomolecule-NP interface. The study can be further simplified by first understanding the interactions of amino acids with Au NP surface, and subsequently with peptides. Methods such as FTIR, ITC and NMR would allow such interactions to be mapped, however hitting the correct concentrations to make such measurements might be an issue.

3) In our quest to investigate protein’s binding orientation and conformation, we have developed a trypsin digestion method coupled with mass spectroscopy. However, given the stochastic nature of the digestion, a large amount of material is required and more specific information such as the order of digestion cannot be deduced. Improvements to the experimental setup can be made by transiting to a single nanoparticle flow cell setup, and peptide fragments flowed out once cleaved. In addition, modifications to proteins, such as mutants or reacting lysines positions with acetic anhydride, can be made to highlight additional folding changes when adsorbed onto Au NPs.
4) New tools are required to more quantitatively and precisely measure protein’s binding orientation and folding. In this aspect, 2D NMR could be a potential solution to a global analysis of protein conformation upon Au NP adsorption. By comparing the spectrum of free versus bound proteins, the change in the local environment of individual amino acids can be detected and quantified. Coupled with the known 3D structure of the protein, the exact binding site as well as conformation change can be deduced.

8.2. Gold Nanoparticle Influences on Cell Migration

The use of Au NPs in therapeutic devices and as theranostic agents is another rapidly developing field that shows great potential for future applications. Our work on Au NPs influencing cellular migration further contributes to this field and reinforces the point that Au NPs can alter the way cells ‘see’ their environment. However, while we have demonstrated that the surface chemistry of Au NPs is important in influencing cell migration, other interesting and potentially impactful questions surfaced from our work.

1) How much of the Au NP uptake by cells occurs at the leading edge, as compared to from the top of the cells? Is the uptake of Au NPs at the leading edge different from uptake from the cell surface? Which cell surface receptors are involved?

2) Why does Au NP size, shape and surface chemistry affects HDF to a greater extend as compared to PC3? How does the cell type influence their interaction with Au NPs?

3) Can Au NP coated surfaces be used for directed cell migration and promote cell alignment?

To answer these questions, a better understanding the molecular interaction of Au NPs with cell surface receptors is necessary. Cell receptors can be selectively blocked or tagged to
obtain insights about the relevant factors in play. Au NPs can be spin coated onto glass substrates prior to cell addition, which affords the advantages of reducing aggregation such that cellular response is more reflective of Au NP properties and controlling surface coverage. By patterning Au NPs on a surface using printing methods, the tendency of cells to track a given Au NP path can be studied.7

8.3. Gold Nanoparticle Influences on Gene Expression and Cellular Pathways

The use of nanoparticles in general has been slowly on the rise. To this date, close to 1000 consumer products boast the use of nanoparticles, and this number is expected to rise in the near future.8 This suggests that humans will be constantly exposed to some quantity of nanoparticles, thus making the understanding of health, safety and environmental implications and risk of high priority. It is therefore critical that the global impact of Au NPs on cells, and not just its purported function, be carefully characterized. To this end, we have used microarray gene analysis to map global gene expression changes of cells after Au NP incubation and have shown that significant changes occur depending on cell type and NP chemistry. However, to extract crucial pattern and pathway changes in cells, further analysis of the data is required. Network mapping would allow for an understanding of how changed genes interaction with each other, and when coupled with functional group analysis can provide insights on prominent cellular functions that are affected.9,10 In addition, our study, as well as others, has only focus on the short term impact of Au NP exposure on cells. In such studies, cells were only incubated with Au NPs for at most 48 h. The long term response of cells to Au NPs (for ~6 months) is thus an equally important, if not more critical, study to map the influence of Au NP exposure on cells. In
a more practical sense, such studies are increasingly becoming more relevant given our potential increased exposure to nanoparticles in the future.

8.4. Final Remarks

The research outlined in this dissertation highlights the importance of understanding the nanoparticle-biomolecular interface and its possible consequence using cell lines. The surface chemistries on Au NPs influences biomolecule interaction and adsorption, which can have interesting and unpredictable effects on cells. It was shown that proteins binding orientation and conformation can differ depending on surface charge on Au NPs, which impacts the protein’s stability and potential function. On a cellular level, functionalized Au NPs influenced cell migration, which can have potential applications in promoting cell alignment and reducing cancer metathesis. The cellular global gene expression change was also studied, demonstrating that cell response to Au NPs depend on cell type and surface chemistries of Au NPs. The knowledge from this work not only contribute to our basis understanding of this complex interaction, but also remind us that we have to be careful and responsible in our applications of Au NPs.
8.5. References


