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NANOPARTICLE-CELL MEMBRANE INTERACTIONS: ADSORPTION KINETICS AND THE MONOLAYER RESPONSE

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NANOPARTICLE-CELL MEMBRANE INTERACTIONS:
ADSORPTION KINETICS AND THE MONOLAYER

RESPONSE

BY

NASIM GANJI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

The fast-growing production and utilization of nanomaterials in diverse applications will undoubtedly lead to the release of these materials into the environment. As nanomaterials enter the environment, determining their interaction with biological systems is a key aspect to understanding their impact on environmental health and safety. It has been shown that engineered nanoparticles (ENPs) can interact with cell membranes by adhering onto their surface and compromising their integrity, permeability, and function. The interfacial and biophysical forces that drive these processes can be examined using lipid monolayers or bilayers as model cell membranes.

Interfacial interactions between NPs and cell membranes have been proven to be affected by various parameters such as the physicochemical properties of the NPs, cell membrane composition, and the extent of exposure. This study focuses on the effects of NP charge, surface functional groups and interfacial activity on the response of lipid monolayers. Dynamic surface pressure measurements were used to examine the kinetics of nanoparticle adsorption and the monolayer response. Fluorescence and real-time *in situ* Brewster angle microscopy (BAM) imaging were employed to characterize the morphology and structure of the monolayers. Bulk concentrations of NP and phosphorus were examined to determine the extent of NP binding and lipid extraction. The results of this study will contribute to further understanding of the membrane's role in ENP cytotoxicity and cellular uptake and aid the design of biocompatible nanomaterials with minimal or controlled membrane activity.

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PREFACE

The following dissertation is presented in manuscript format. This dissertation is a compilation of two published manuscripts and one manuscript under preparation.

Chapter 2 entitled “Anionic and Cationic Silver Nanoparticle Binding Restructures Net-Anionic PC/PG Monolayers with Saturated or Unsaturated Lipids” has been published in *Langmuir* in 2017, volume 33, pages 353-360.

Chapter 3 entitled “Surface Activity of Poly(Ethylene Glycol)-Coated Silver Nanoparticles in the Presence of a Lipid Monolayer” has been published in *Langmuir* in 2018, volume 34, pages 2039-2045.

Chapter 4 entitled “Human serum protein coronas alter interactions between nanoparticles and a model red blood cell membrane” has been prepared to be submitted to *Nanoscale*.

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CHAPTER 1

INTRODUCTION

The production and utilization of engineered nanoparticles (ENPs) in technology and medicine is constantly expanding;¹ however, there are still many uncertainties associated with the potential risks that they pose to environmental health and safety (EHS).^{2,3} Fundamental studies that assess the hazard of ENPs are necessary in order to promote safe use and limit risks, and to guide the design of environmentally and biologically compatible materials.⁴

Due to their high specific surface area and nanoscale size (<100 nm), ENPs display novel physical and chemical properties that are substantially different from those observed in the bulk materials.^{5,6} Hence, ENPs are suitable candidates for a broad variety of commercial applications. For instance, metal NPs such as gold⁷ or silver^{9,10} exhibit unique optical, electronic and catalytic properties, primarily due to their localized surface plasmon resonance (LSPR) characteristics,¹¹ and they have been used for environmental remediation, (bio)chemical sensing, and drug delivery.^{6,12} However, nanoparticles have been shown to bioaccumulate and exhibit various levels of toxicity.^{13–15} This can be attributed to their size, shape, surface chemistry, and surface reactivity, which may allow them to penetrate tissues, enter cells, and interact with the compartments of the cell membrane.^{16,17} This process can lead to a range of nanoparticle-induced biophysical and/or biochemical changes with the degree of change dependent on a variety of parameters such as cell membrane composition, NPs

concentration and physicochemical properties, and the extent of exposure.^{18–22} As a result, the safe use of ENPs in biological systems requires evaluation of their possible cytotoxicity.

Recent toxicological studies conducted *in vitro* and *in vivo* have demonstrated that both carbon-based^{13,14} and inorganic^{23,24} NPs can strongly interact with cell membranes, and cause cytotoxicity through a variety of disruptive mechanisms including (1) adherence of the NPs to the membrane, (2) aggregation around the membrane, (3) removal of lipids from the membrane, and (4) permanently embedding into the membrane.²⁴ Adhesive forces between nanoparticles and cell surfaces driven by surface interactions, notably electrostatic, hydrophobic, and van der Waals, govern the timescale for nanoparticle-cell association, membrane disruption, and the extent of cellular uptake.^{25–27} This behavior is independent of well-known cytotoxicity mechanisms related to chemical stability by which inorganic ENPs can release ions into solution or generate reactive oxygen species.²⁸ Dawson et al.²⁹ described how the scientific community generally views nanoparticle-cell interactions as occurring through “classical biological processes,” but emphasized the importance of physical interactions (thus far neglected), such as those occurring between nanoparticles and membrane barriers. This is further emphasized by observations that greater nanoparticle-lipid interactions correlate with greater cellular uptake.^{30,31} Hence, understanding nanoparticle-membrane interactions at the biophysical level will provide new insight into how nanoparticles affect cell function and viability. Understanding these interactions will elucidate the membrane’s role in ENP cytotoxicity and cellular uptake and aid the design of biocompatible nanomaterials. This increased understanding

may also provide new routes for designing nanoscale assemblies for biomedical applications.

NP uptake initiates with an attachment of the particle to the cell and subsequent interactions with the lipids and other components of the cell membrane. The interfacial and biophysical interactions that modulate this process can be examined using lipid bilayers or monolayers as model cell membranes.^{27,32–40}

Cellular membranes are complex, multicomponent systems that contain a variety of charged and uncharged lipids with different degrees of tail saturation. In model cell membranes, attempts to mimic the complexity of real membranes involve adding multiple lipids to achieve a net surface charge and/or coexisting membrane domains (e.g., ordered and disordered). Two main advantages of model membranes are that the lipid composition can be varied, and that membrane organization and disruption can be measured directly using techniques that are not amenable to living cells. These simplified structures can be considered as first step approaches to investigate real systems due to their ability to mimic some of the most relevant physicochemical features of the real cell membrane.^{27, 34}

The overall objectives of this dissertation were (1) to develop experimental approaches to capture the key parameters that control the duration and extent of nanoparticle adhesion to model cell membranes, and (2) to quantify physical nanoparticle-cell membrane interactions as a function of nanoparticle interfacial properties and cell membrane composition, and to determine how local NP-cell membrane interactions yield global changes in cell membrane structure and function.

This project has been accomplished using lipid monolayers as model cell membranes and environmentally relevant nanoparticles and surface coatings; and by extending biophysical membrane concepts to nanoparticle interactions.

Chapter 2 focuses on the effects of AgNP charge, provided by anionic and cationic polymer coatings, on the response of two-component monolayers composed of saturated or unsaturated phosphocholine (PC)/phosphoglycerol (PG) lipids. This study contributes to further understanding of how lipid phase behavior and inter-lipid interactions, which depend on lipid composition, modulate the nature and extent of nanoparticle–membrane interactions.

Chapter 3 evaluates the kinetics of poly(ethylene glycol)-coated silver (Ag-PEG) nanoparticle adsorption at the air-water interface, the degree of monolayer coverage, and how the presence of lipid monolayers changes these properties. The aim of this study was to highlight the role of hydrophobic interactions in NP adsorption or penetration into lipid monolayers.

Finally, Chapter 4 investigates the response of human red blood cell model membranes to the adhesion of polystyrene (PS) nanoparticles with a particular emphasis on the effect of NP surface chemistry and protein corona formation on this process.

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CHAPTER 2

Anionic and Cationic Silver Nanoparticle Binding Restructures Net-Anionic PC/PG Monolayers with Saturated or Unsaturated Lipids

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ABSTRACT

We have examined the interactions between polymer-coated anionic (Ag-COOH) and cationic (Ag-NH) silver nanoparticles, and net-anionic lipid monolayers using dynamic surface pressure measurements. Monolayers composed of saturated or mono-unsaturated mixtures of anionic phosphatidylglycerol (PG) and zwitterionic phosphatidylcholine (PC) lipids (3:1 molar ratio) were used to determine how lipid packing and monolayer phase state influence the extent of nanoparticle binding and the monolayer response. Anionic Ag-COOH inserted into saturated dipalmitoyl-PC/PG (DPPC/DPPG) and dioleoyl-PC/PG (DOPC/DOPG) monolayers at a low initial surface pressure (10 mN m^{-1}) and caused lipid condensation at high initial surface pressures (20 and 30 mN m^{-1}). Hydrophobic interactions were responsible for insertion, while electrostatic and charge-dipole interactions with PCs were responsible for condensation. In contrast, cationic Ag-NH inserted only into saturated DPPC/DPPG monolayers and otherwise led to lipid condensation. For Ag-NH, adsorption was driven primarily by electrostatic interactions with PGs. Analysis of the subphase Ag and phosphorus concentrations confirmed that Ag-NH had a higher degree binding compared to Ag-COOH, and that the monolayer response was not due to lipid extraction.

INTRODUCTION

Physical interactions between engineered nanoparticles and lipid membranes play an important role in nanotoxicology and nanomedicine.¹⁻³ Adhesive forces between nanoparticles and cell surfaces driven by surface interactions, notably electrostatic, hydrophobic, and van der Waals interactions, govern the timescale for nanoparticle-cell association,⁴ changes in nanoparticle organization at the membrane/water interface,⁵⁻⁶

membrane disruption,⁷ and the extent of and cellular uptake.⁸⁻⁹ The interfacial and biophysical interactions that drive these processes can be examined using lipid bilayers or monolayers as model cell membranes.¹⁰⁻¹⁸ The main advantages of model membranes are that the lipid composition can be varied and that membrane organization and disruption can be measured directly using techniques that are not amenable to living cells. Model membranes have been used extensively to examine the adsorption of, and in some cases the resulting disruption caused by, carbonaceous,¹⁹⁻²² metal oxide,²³⁻²⁹ metallic,^{11, 30-32} and polymeric^{28, 33} nanoparticles. Recent studies have also been conducted to determine how proteins or natural organic matter, adsorbed onto the nanoparticle surface, influence membrane interactions.^{20, 34}

Cellular membranes are complex, multicomponent systems that contain a variety of charged and uncharged lipids with varying degrees of tail saturation. In model cell membranes, attempts to mimic this complexity involve adding multiple lipids to achieve a net surface charge and/or co-existing membrane domains (e.g. ordered and disordered). In context of nanoparticle-membrane interactions, Ha et al.¹⁹ have shown that fullerene partitioning to lipid bilayers composed of biologically relevant ternary lipid mixtures that can form liquid ordered ‘lipid raft’ domains is lower below the phase transition temperature than above the transition temperature when the rafts are present. Increased interfacial area (area per lipid) when rafts were present increased fullerene partitioning. Earlier work comparing the interactions between polystyrene nanoparticles and a model endothelial membrane (EM) monolayer to the individual lipid components showed that the response of model EM monolayer was unique, demonstrating the importance of interlipid interactions and how they influence nanoparticle interactions.³³

Similar work by Guzman et al.^{24, 35} using a mixed lipid monolayer composed of a saturated and an unsaturated lipid showed that anionic silica nanoparticles bound to both lipids and inserted into the monolayer, but limited the formation of condensed saturated lipid domains and increased lipid miscibility within the monolayer. Studies such as these demonstrate the important and still illusive role of lipid phase behavior and interlipid interactions, which depend on lipid composition, in modulating the nature and extent of nanoparticle-membrane interactions.

In this work, we have examined the response of two-component monolayers composed of saturated or unsaturated phosphocholine (PC)/phosphoglycerol (PG) lipids to anionic and cationic silver nanoparticles (AgNPs). Cationic nanoparticles have been shown to bind quickly and strongly to PC/PG membranes leaving them intact, but causing an increase in membrane rigidity.³⁶ Cationic nanoparticle binding to PC and PC/PG membranes also lead to membrane protrusions and pore formation due to ‘steric crowding’ within the membrane as the nanoparticles pack on the surface and consume excess area between the lipids.¹² Steric crowding caused the lipids to pack more tightly or compress, which increased the surface tension of the membrane. Finally, we have also shown that anionic and cationic silver nanoparticles (AgNPs) bind to PC/PG membranes (bilayer vesicles) without membrane rupture.¹¹ However, AgNP binding did lead to membrane deformation and vesicle aggregation due to membrane-AgNP-membrane bridging.¹¹

Lipid monolayers have been successfully used to examine nanoparticle-lipid interactions based on changes in interlipid interactions that affect the degree of lipid packing and the monolayer phase behavior, and on lipid extraction from the air/water

interface due to nanoparticle-lipid binding.^{23-31, 33, 37-38} This study focuses on the effects of AgNP charge, provided by anionic and cationic polymer coatings (Fig. 2-1), on the response of PC/PG monolayers (3:1 mol). Dynamic surface pressure measurements were used to examine the duration and extent of nanoparticle adsorption and the monolayer response. Sub-phase Ag and phosphorus (P) concentrations were examined to confirm AgNP binding and the extent of lipid extraction.

MATERIALS AND METHODS

Materials. PC and PG lipids were obtained from Avanti Polar Lipids (Alabaster, AL) and nitric acid (65-71%, TraceSELECT Ultra grad), standard Ag solution (1000 mg L⁻¹ in nitric acid, TraceCERT® grade), and standard P solution (1000 mg L⁻¹ in H₂O, TraceCERT® grade) were purchased from Sigma-Aldrich (St. Louis, MO). Anionic and cationic AgNPs were obtained from Ocean NanoTech (San Diego, CA). Lipids and AgNPs were used as received. Schematics of the AgNPs are shown in Fig. 2-1. Anionic AgNPs, referred to as Ag-COOH, were coated with a carboxylated amphiphilic polymer formed by hydrolyzing poly-(maleic anhydride-alt-1-octadecane).³⁹⁻⁴⁰ Cationic AgNPs, referred to as Ag-NH, were prepared by coating Ag-COOH nanoparticles with polyethyleneimine. Sterile, ultra-filtered deionized water was obtained from Millipore Direct-3Q purification system and adjusted to pH 7.

Figure 2-1 Schematic of anionic (Ag-COOH) and cationic (Ag-NH) silver nanoparticles (not to scale).

Nanoparticle characterization. AgNP size was determined by transmission electron microscopy using a JEOL JEM-2100F TEM (Peabody, MA) operating at 200 keV. The reported average core radius (r_c) is based on size analysis of over 200 nanoparticles obtained from multiple images using the software program ImageJ software.⁴¹ Hydrodynamic radii (r_h) and zeta potential (ζ) were measured using a Malvern Instruments Zetasizer Nano ZS with a backscattering detector angle of 173° and a 4 mW, 633 nm He-Ne laser (Worcestershire, UK). Samples diluted with DI water were placed in an optical grade polystyrene cuvette at 25 °C. The average z-averaged hydrodynamic radii and zeta potentials reported are based on triplicate measurements each with 15 scans. Zeta potential was measured by combined Doppler electrophoretic velocimetry and phase analysis light scattering, and values were obtained based on Smoluchowski theory.

Monolayer surface pressure measurements. Monolayer experiments were conducted at 20 °C in three sequential steps: (1) surface pressure–area (π –A) isotherm measurements of lipid monolayers in the absence of AgNPs, (2) dynamic surface pressure measurements at selected initial surface pressures ($\pi_0 = 10, 20,$ and 30 mN m^{-1}) in the presence of AgNPs, and (3) analysis of Ag and phosphorus (P) concentrations in the subphase using ultraviolet-visible (UV-vis) spectroscopy and/or inductively coupled plasma mass spectroscopy (ICP-MS) (Fig. 2-2).

Figure 2-2 (A) Schematic of the Langmuir trough system to measure dynamic changes in monolayer surface pressure due to lipid-nanoparticle binding. (B1-B3) Possible modes of interaction leading to changes in surface pressure (not to scale).

Step (1) was used to stabilize the monolayer and determine lipid packing as a function of surface pressure, expressed as the area per lipid molecule (A , $\text{\AA}^2 \text{ molecule}^{-1}$), and lipid phase state. Charge density (charge nm^{-2}) was calculated from A based on 25 mol% DPPG or DOPG. Monolayers were prepared in a Teflon® Langmuir-Blodgett trough (model 102M, KSV NIMA, Biolin Scientific Inc., Linthicum Heights, MD) by spreading dissolved lipids in chloroform at the air/water interface and allowing 30 min for the chloroform to evaporate. The water subphase volume within the trough was approximately 140 mL. While the experiments were conducted in deionized water, DPPG and DOPG are sodium salts and the concentration of Na^+ counterions within the subphase was equivalent to 3×10^{-5} mM. Isotherms were generated for a single compression/expansion cycle at a barrier rate of $10 \text{ cm}^2 \text{ min}^{-1}$ and π was measured using paper Wilhelmy plates. The total area of the trough during this cycle ranged from roughly 20–70 cm^2 .

Step (2) was used to determine the change in monolayer surface pressure in the presence of AgNPs as a function of time. To measure dynamic changes in surface pressure ($\Delta\pi$) the trough was initially set to maintain a constant surface pressure ($\pi_0 = 10, 20, \text{ or } 30 \text{ mN m}^{-1}$) after the compression/expansion isotherms (step 1). Once the monolayer stabilized and π_0 remained constant, the barrier positions were fixed at the corresponding interfacial area or charge density. AgNPs were added to the water subphase by injecting them behind the barriers using a syringe to avoid disrupting the monolayer. The volume and concentration of the AgNP solution that was injected was 100 μL and 5 mg mL^{-1} , respectively. The AgNPs were mixed within the subphase by recycling the solution using a peristaltic pump. Control experiments confirmed that the

pumping action did not disturb the monolayers and that water evaporation did not alter the $\Delta\pi$ measurements. The initial AgNP concentration in the subphase was 3.6 mg L⁻¹ or 33.4 μ M, which was estimated to provide excess surface coverage based on the AgNP cross sectional area at a monolayer surface area of 70 cm².

Step (3) was used to determine the subphase concentrations of Ag and P, [Ag] and [P] respectively. Sample volumes of 1 mL were removed from the subphase behind the barriers after step (2). AgNP plasmon resonance absorption was measured by UV-vis spectroscopy (Cary 50, Varian, Palo Alto, CA) and based on the maximum peak height at wavelengths of 410 nm after baseline subtraction. For [Ag] and [P] determined by ICP-MS (iCAP Q, Thermo Scientific, Waltham, MA), samples were digested using nitric acid and then diluted 10 times with DI water. Standard solutions containing 0.1, 1, 10, 100 and 1000 μ g L⁻¹ of Ag and P were used for instrument calibration. Trace amounts of Ag and P measured in deionized water and in the acid digestion solution were subtracted from the reported values. All measurements were conducted in triplicate.

RESULTS

AgNP Characterization. AgNPs were characterized prior to the monolayer experiments to confirm their physicochemical properties and to determine the extent of AgNP dissolution. The average r_c was 6 ± 2 nm based on TEM analysis and was common to both Ag-COOH and Ag-NH (TEM, Fig. 2-3A). The polymer coatings surrounding the AgNPs were not observed in the micrographs. Ag-COOH had a hydrodynamic radius, r_h , of 14 ± 2 nm (0.02 PDI) and a zeta potential, ζ , of -63 ± 3 mV. Ag-NH had a $r_h = 20 \pm 3$ nm (0.02 PDI) and a $\zeta = +46 \pm 2$ mV. The average coating

thicknesses based on the difference between r_h and r_c were 8 nm for Ag-COOH and 14 nm for Ag-NH. The increase in coating thickness from Ag-COOH to Ag-NH is consistent with PEI coating of Ag-COOH.

The maximum absorbance due to AgNP surface plasmon resonance (SPR) was observed at a wavelength of 410 nm (Fig. 2-3B). The SPR absorbance was measured over 3 months to confirm the stability of the AgNPs and determine the extent of dissolution. There was no shift in the SPR wavelength, indicating that the AgNPs were stable. A slight reduction in SPR absorbance was observed over 3 months consistent with a ~3% decrease in the AgNP concentration. Given that the monolayer studies were conducted within 1 month of receiving the samples, we did not account for AgNP dissolution in our analyses.

Finally, the surface activity of the native AgNPs was examined in the absence of a lipid monolayer (Fig. 2-3C). The π -A isotherm for Ag-COOH and Ag-NH showed a π of 16.9 mN m⁻¹ and 6.3 mN m⁻¹, respectively, with 74% compression (70 to 18 cm²) indicating that polymer coatings rendered the nanoparticles surface active due to hydrophobic interactions at the air/water interface.

Figure 2-3 (A) Histogram plot of AgNP core radii, r_c , based on TEM analysis (a representative micrograph is shown inset). (B) UV-vis spectra of Ag-COOH and Ag-NH over 3 months. (C) Surface pressure-area isotherm for AgNPs at 20 °C.

Lipid monolayer π -A isotherms. The chemical structures of the lipids are shown in Fig. 2-4 (top) and surface pressure–area isotherms are shown in Fig. 2-4A for DPPC/DPPG (saturated tails) and DOPC/DOPG (mono-unsaturated tails) monolayers (step 1). Increasing π corresponded to a decrease in A with compression as the lipids packed more tightly at the interface. As shown in Fig. 2-4B, increasing π corresponded to an increase in monolayer charge density that was calculated based on the number of charged PG lipids at the interface divided by the trough area at the respective π . DPPC/DPPG monolayers existed as liquid-expanded (LE) phases at 10 mN m⁻¹, mixed LE–liquid-condensed (LC) phases at 20 mN m⁻¹ (based on the inflections between 18 and 25 mN m⁻¹), and as LC phases at 30 mN m⁻¹. DPPC monolayers display similar phase behavior due to their saturated tails that allow them to pack tightly to form a LC phase at high surface pressures, however mixed LE-LC phases are typically observed at lower surface pressures.⁴² DOPC/DOPG monolayers existed as LE phases consistent with the behavior of pure DOPC.⁴² DOPC and DOPG have monounsaturated acyl tails that are ‘kinked’ around the C=C double bonds in each tail and restrict tight packing.

Dynamic changes in monolayer surface pressure due to AgNP adsorption.

Dynamic changes in monolayer surface pressure, $\Delta\pi$, were determined as $\Delta\pi = \pi(t) - \pi_0 = \gamma_0 - \gamma(t)$, where $\pi(t)$ is the dynamic surface pressure after AgNP addition and π_0 is the initial surface pressure of the air/lipid/water interface. The relationship between $\Delta\pi$ and the initial air/lipid/water interfacial tension, γ_0 , and the dynamic interfacial tension, $\gamma(t)$, shows that an increase in $\Delta\pi$ would result from a decrease in $\gamma(t)$ due to AgNP-lipid monolayer interactions (and vice versa). Changes in $\Delta\pi$ are depicted in Fig. 2-2 B1-B3

for the proposed AgNP-lipid monolayer interaction mechanisms. Hädicke and Blume⁴³ have shown that dynamic surface measurements with cationic peptides and anionic DPPG monolayers can be used to differentiate between peptide insertion into the monolayer (increasing $\Delta\pi$) and lipid condensation due to peptide-lipid binding (decreasing $\Delta\pi$). This approach has also been used to examine the insertion of gold nanoparticles into DPPC monolayers.³⁰

Figure 2-4 (A) Surface pressure-area (π -A) isotherms for DPPC/DPPG (3:1) and DOPC/DOPG (3:1) monolayers at 20 °C and (B) monolayer surface charge density at $\pi = 10, 20$, and 30 mN m^{-1} , which correspond to π_0 for the dynamic surface pressure measurements. Error bars for (B) based on three independent isotherms. Chemical structures of the lipids are shown above the graphs.

Anionic Ag-COOH. Changes in surface pressure for DPPC/DPPG and DOPC/DOPG monolayers due to Ag-COOH adsorption (step 2) are shown in Fig. 2-5A1 and 5B1, respectively, at $\pi_0 = 10, 20,$ and 30 mN m^{-1} . Fig. 2-5A1 and 5B1 show duplicate experiments for $\Delta\pi$ as a function of time, and Fig. 2-5A2 and 5B2 show $\Delta\pi$ as a function of π_0 at select times (30, 60, 90, and 180 min) with vertical arrows depicting the increase or decrease in $\Delta\pi$ over time. The bilayers responded similarly to Ag-COOH adsorption, displaying a slight increase in $\Delta\pi$ when the lipids were loosely packed at $\pi_0 = 10 \text{ mN m}^{-1}$ and decreases in $\Delta\pi$ at $\pi_0 = 20$ and 30 mN m^{-1} . This suggests that the Ag-COOH nanoparticles are able to insert into the monolayer when the lipids are loosely packed (Fig. 2-2B1) – when loosely packed, inter-lipid interactions are weak and there is excess ‘free area’ at the air/water interface. The increase in $\Delta\pi$ is due to a reduction in interfacial tension as the lipids pack to accommodate the nanoparticles. Overall, the duration over which $\Delta\pi$ changed for DPPC/DPPG and DOPC/DOPG shows that the monolayer response due to nanoparticle-lipid interactions occurred over long time scales up to 180 min, which is consistent with previous work with hydrophilic³⁰ or hydrophobic²⁷ nanoparticles.

Increased initial packing (based on π_0) prevented nanoparticle insertion and the decrease in $\Delta\pi$ indicates that Ag-COOH led to lipid condensation (Fig. 2-2B2). This behavior was independent of phase state. A linear fit of $\Delta\pi$ as a function π_0 at $t = 180$ min was used to estimate the minimum insertion pressure (MIP) of Ag-COOH, which corresponds to the condition $\Delta\pi = 0$ (Figures 2-5A2 and 5B2). We refer to insertion as meaning that the nanoparticles breach the plane of the monolayer and occupy area at the air/water interface with or without an adsorbed lipid coating. The MIPs for

DPPC/DPPG and DOPC/DOPG were 13.9 and 12.1 mN m⁻¹, respectively, indicating that below this surface pressure the nanoparticles are capable of inserting into the monolayer. Above the MIP inter-lipid interactions within the monolayer resist nanoparticle insertion. The MIPs determined for Ag-COOH are considerably lower than those reported for 10 and 15 nm diameter anionic gold nanoparticles and zwitterionic DPPC monolayers.³⁰ It should be noted that the gold nanoparticle concentration was more than order of magnitude higher than what was used in this work.

Figure 2-5 Duplicate experiments depicting dynamic changes in surface pressure, $\Delta\pi$, for DPPC/DPPG (A1) and DOPC/DOPG (B1) monolayers as a function of time after the addition of anionic Ag-COOH. Results for $\Delta\pi$ as a function of initial surface pressure, π_0 , and time (30, 60, 90, 180 min) are shown for DPPC/DPPG (A2) and

DOPC/DOPG (B2). The initial monolayer phase states shown in A2 and B2 are based on Fig. 2-4, and vertical errors denote the change in $\Delta\pi$ with time. Standard error bars shown in A2 and B2 are based on A1 and B1, respectively.

We now consider the factors that may have facilitated the interaction between anionic Ag-COOH nanoparticles and anionic monolayers. The experiments were conducted in DI water and as a result one would expect strong electrostatic repulsion between Ag-COOH and the monolayers. Three modes of interaction are possible: hydrophobic interactions with lipids tails, counterion-mediated (Na^+) binding to PGs, and electrostatic and charge-dipole interactions with PCs. The surface activity of Ag-COOH supports the assertion that Ag-COOH penetrated into loosely packed monolayers at $\pi_0 = 10 \text{ mN m}^{-1}$ and resided at the air/water interface. It should be noted that the ability for Ag-COOH to insert into the monolayer might also stem from the nanoparticles being rendered partially hydrophobic due to the adsorption of lipids at the air/water interface and the formation of nanoparticle-lipid complexes.²⁴ Hydrophobic interactions do not, however, explain the reductions in surface pressure at 20 or 30 mN m^{-1} .

With regards to counterion-mediated binding, the Na^+ counterions associated with PGs may have facilitated the adsorption of Ag-COOH. This mode of adsorption has been proposed for anionic citrate-coated gold nanoparticles and DPPG monolayers, which caused an increase in surface pressure (or monolayer expansion).³¹ Given that PGs comprised only 25 mol% of the monolayers examined herein, and significant

decreases in surface pressure were observed consistent with lipid condensation, it is unlikely that counterion-mediating adsorption played a dominant role.

Electrostatic and charge-dipole interactions with PCs, which were present at 75 mol% in the monolayers, appear to be a main driving force for Ag-COOH adsorption. At $\pi_0 = 20$ and 30 mN m^{-1} the reductions in surface pressure suggest that the nanoparticles did not penetrate the monolayer, but rather remained bound to the monolayer below the interface and caused lipid condensation (i.e. a reduction in the effective area per lipid). It has been shown that anionic nanoparticles can bind to zwitterionic lipids⁴⁴ and pulmonary surfactant monolayers²⁸ through attractive interactions with the positive choline group of zwitterionic lipids.²³⁻²⁵ Zwitterionic lipids have a dipole moment extending into the aqueous phase that can also lead to attractive short-range ion-dipole interactions. Anionic nanoparticles can reorient the headgroup dipoles of zwitterionic lipids, causing the dipole to orient perpendicular to the lipid/water interface and reducing the area per lipid.⁴⁴ Hence, lipid condensation in the monolayers appears to be attributed to the dipole reorientation of DPPC and DOPC. The ability for Ag-COOH to adsorb onto DPPC/DPPG monolayers is consistent with our previous work showing Ag-COOH adsorption onto DPPC/DPPG bilayer vesicles.¹¹

The role of lipid condensation was examined further using monolayers containing equimolar mixtures of PC and PG lipids (data not shown). Reducing the concentration of DPPC or DOPC from 75 mol% to 50 mol% reduced the magnitude of the $\Delta\pi$ decrease. With less PC lipid there was less lipid condensation.

Cationic Ag-NH. In contrast to Ag-COOH, the monolayers responded differently to oppositely charged Ag-NH and MIP values could not be determined (Fig. 2-6). A

significant increase in $\Delta\pi$ was observed for DPPC/DPPG monolayers at $\pi_0 = 10 \text{ mN m}^{-1}$ consistent with Ag-NH insertion into the monolayer (Fig. 2-6A1). At $\pi_0 = 20 \text{ mN m}^{-1}$ a decrease in $\Delta\pi$ was observed suggesting that lipid condensation occurred, and at $\pi_0 = 30 \text{ mN m}^{-1}$ a two-state response was observed where $\Delta\pi$ increased rapidly up to 10 min (insertion) and then decreased exponentially (condensation). The rapid increase in $\Delta\pi$ observed initially at $\pi_0 = 10$ and 30 mN m^{-1} was due to electrostatic attraction between the monolayers and Ag-NH that drove adsorption and insertion. Electrostatic attraction was also present at $\pi_0 = 20 \text{ mN m}^{-1}$, however, the surface pressure response reflected competition between lipid condensation and Ag-NH insertion, where at this initial surface pressure, lipid condensation had the greatest impact on $\Delta\pi$. For DOPC/DOPG, $\Delta\pi$ was unchanged (10 mN m^{-1}) or reduced (20 and 30 mN m^{-1}) and there was no evidence of Ag-NH insertion. Only lipid condensation was observed at high initial surface pressures. Lipid condensation caused by cationic Ag-NH was driven by electrostatic attraction with anionic DPPG or DOPG lipids and inter-lipid charge neutralization. This differs from anionic Ag-COOH, which interacted with the zwitterionic lipids. Previous work has shown that cationic gold nanoparticles have a minimal effect on the surface pressure isotherms of DPPC,³¹ which further supports the assertion that PGs were responsible for Ag-NH adsorption.

Figure 2-6 Duplicate experiments depicting dynamic changes in surface pressure, $\Delta\pi$, for DPPC/DPPG (A1) and DOPC/DOPG (B1) monolayers as a function of time after the addition of cationic Ag-NH. Results for $\Delta\pi$ as a function of initial surface pressure, π_0 , and time (30, 60, 90, 180 min) are shown for DPPC/DPPG (A2) and DOPC/DOPG (B2). The initial monolayer phase states shown in A2 and B2 are based on Fig. 2-4, and vertical errors denote the change in $\Delta\pi$ with time. Standard error bars shown in A2 and B2 are based on A1 and B1, respectively.

Subphase analysis of AgNP and lipid (phosphorus) concentration. The subphase concentrations of AgNPs or Ag and P (one P per lipid) were analyzed to determine if the monolayer responses were attributed to differences in amount of AgNP adsorbed or

the extraction of lipids from the monolayer (Fig. 2-2B3), respectively. Results are shown for AgNPs analyzed by UV-vis (SPR; Fig. 2-7A) and ICP-MS ([Ag]; Fig. 2-7B) as a function of monolayer charge density. The sub-phase concentrations represent the total amount of AgNPs or lipid in the system minus the amount of AgNPs or lipid at the air/water interface. There was good agreement between the trends in UV-vis absorbance and [Ag] for each AgNP and monolayer. ICP-MS analysis of sub-phase [P] showed that the lipid extraction ranged from 0.1-6% with no clear relation to charge density (results not shown). Most of the [P] results were not significantly different based on standard error. Therefore, we conclude that lipid extraction was not a significant factor that led to decreases in $\Delta\pi$ observed in Figures 2-5 and 2-6.

The concentration of AgNPs in the sub-phase (Fig. 2-7) provides a number of insights into the monolayer response. First, there is generally little difference in AgNP concentrations between the two monolayers; the exception being Ag-COOH at the highest monolayer charge density where the standard errors were large. This suggests that AgNP adsorption was primarily driven by lipid headgroup interactions and that the monolayer response was driven by the lipid tail saturation and phase behavior. Second, the sub-phase concentration of Ag-NH is less than Ag-COOH, which means that more cationic Ag-NH nanoparticles were bound to the anionic lipid monolayers. Mass balances based on the [Ag] results show that 15-21% of Ag-NH and 24-47% of Ag-COOH remained in the sub-phase. The low amount of P (lipid) detected in the sub-phase, and our previous work examining interactions between AgNPs and lipid vesicles, indicates that the AgNPs remaining in the subphase did not contain lipid coatings (monolayers or bilayers). Third, the sub-phase concentrations were independent of

monolayer charge density within the standard error. In DI water the Debye screening length is on the order of 800 nm (neglecting Na^+ counterions associated with the PGs) and strong electrostatic interactions persisted across the range of monolayer charge densities. Additional work is needed to determine AgNP adsorption and the monolayer response as a function of salt concentration to examine the effects of electrostatic interactions.

CONCLUSIONS

Previous studies have shown that nanoparticles interact with model membrane bilayer and monolayers, and our work indicates that many of the interactions mechanisms reported for single lipids are conserved in PC/PG lipid mixtures. Anionic Ag-COOH nanoparticles penetrate into monolayers via hydrophobic interactions and bind to zwitterionic lipids and cause condensation, but the presence of an anionic lipid appears to lessen this interaction via electrostatic repulsion when compared to previous work using similarly sized gold nanoparticles.³⁰⁻³¹ This behavior was observed in both saturated DPPC/DPPG and unsaturated DOPC/DOPG monolayers. Cationic Ag-NH nanoparticles adsorb through electrostatic attraction with PG, and drive monolayer penetration into saturated, but not unsaturated lipid monolayers. Furthermore, there was evidence of nanoparticle penetration and lipid condensation in saturated monolayers whereas unsaturated monolayers only exhibited lipid condensation. In general, unsaturated monolayers exhibited greater lipid condensation compared to saturated monolayers due to the larger area per lipid stemming from the lipid tails.

Figure 2-7 Subphase Ag concentrations for Ag-NH (Fig. 2-5) and Ag-COOH (Fig. 2-6) below the PC/PG monolayers based on (A) UV-vis plasmon resonance absorbance at maximum peak height for AgNPs and (B) ICP-MS.

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CHAPTER 3

Surface activity of poly(ethylene glycol)-coated silver nanoparticles in the presence of a lipid monolayer

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ABSTRACT

We have investigated the surface activity of poly(ethylene glycol) (PEG)-coated silver nanoparticles (Ag-PEG) in the presence or absence of lipid monolayers comprised of mono-unsaturated dioleoylphosphocholine and dioleoylphosphoglycerol (DOPC/DOPG; 1:1 mole ratio). Dynamic measurements of surface pressure demonstrated that Ag-PEG were surface-active at the air/water interface. Surface excess concentrations suggested that at high Ag-PEG subphase concentrations, Ag-PEG assembled as densely-packed monolayers in the presence and absence of a lipid monolayer. The presence of a lipid monolayer led to only a slight decrease in the excess surface concentration of Ag-PEG. Surface pressure-area isotherms showed that in the absence of lipids, Ag-PEG increased the surface pressure up to 45 mN m^{-1} upon compression before the Ag-PEG surface layer collapsed. Our results suggest that surface activity of Ag-PEG was due to hydrophobic interactions imparted by a combination of the amphiphilic polymer coating and the hydrophobic dodecanethiol ligands bound to the Ag-PEG surface. With lipid present, Ag-PEG + lipid surface pressure-area (π -A) isotherms reflected Ag-PEG incorporation within the lipid monolayers. At high Ag-PEG concentrations, the π -A isotherms of the Ag-PEG + lipid films closely resembled that of Ag-PEG alone, with a minimal contribution from the lipids present. Analysis of the subphase silver (Ag) and phosphorus (P) concentrations revealed that most of the adsorbed material remained at the air/lipid/water interface and was not forced into the aqueous subphase upon compression, confirming the presence of a composite Ag-PEG + lipid film. While interactions between ‘water-soluble’ nanoparticles and lipids are often considered to be dominated by electrostatic

interactions, these results provide further evidence that the amphiphilic character of a nanoparticle coating can also play a significant role.

INTRODUCTION

Engineered metal NPs exhibit distinctive physicochemical properties and thus have been studied in diverse research fields such as (bio)chemical sensing, multifunctional catalysis, and drug delivery.¹⁻⁹ Among them, engineered silver nanoparticles (AgNPs) are well-known for their high electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity, and non-linear optical behavior; and have recently been developed for use in medical imaging and bio-sensing.¹⁰⁻¹² The safe use of AgNPs in living systems requires evaluation of their possible cytotoxicity. Recent studies have revealed that inorganic engineered NPs (ENPs) such as silver can strongly interact with cell membranes¹³⁻¹⁷ causing cytotoxicity through a variety of disruptive mechanisms including (1) adherence of the NPs to membrane, (2) aggregation around the membrane, (3) removal of lipids from membrane, and (4) permanently embedding into membrane.¹⁸ Interfacial interactions between ENPs and cell membranes are proved to be affected by various parameters such as the physicochemical properties of the ENPs (specially surface charge, hydrophobicity, size, shape and surface functionality), cell membrane composition, and the extent of exposure.¹⁹⁻²¹

Lipid monolayers or bilayers employed as model membranes can be considered a first step to investigating ENP-membrane interaction mechanisms due to their ability to mimic many relevant physicochemical features of cell membranes.^{22,23} Langmuir monolayers, in particular, have been used as an effective tool for characterizing specific

interactions at the molecular-level with membrane-forming lipids.^{12,20,24,25} The surface pressure-area (π - A) isotherms of the Langmuir films reveal the intermolecular forces operating in two dimensions (2D) as well as information on the arrangement and orientation of lipids. Our previous study has addressed the effects of AgNP charge, provided by anionic and cationic polymer coatings, on the duration and extent of AgNP adsorption and the response of PC/PG monolayers (3:1 mol). Dynamic surface pressure measurements revealed that AgNP binding restructures monolayers at air/water interface, with anionic AgNPs inserting into net-anionic monolayers via hydrophobic interactions and cationic AgNP adsorbing through electrostatic attraction with PG.²⁶

The Langmuir-Blodgett (LB) technique is further useful for studying the surface activity of amphiphilic polymer-coated NPs at interfaces that drives interfacial adsorption. Like surfactants and lipid molecules, these capped NPs self-assemble into 2D lattices at air/water interfaces.^{27,28} Here, we characterize the surface activity of Ag-PEG in the presence or absence of net-anionic lipid monolayers (DOPC/DOPG; 1:1 mol). PEG has been the focus of research as an effective coating material due to its biocompatibility and long period of circulation in the bloodstream.^{29–33} Even though PEG is completely water-soluble at room temperature, PEG of sufficiently high molecular weight can form Langmuir monolayers at the air/water interface.³⁴ Early work analyzing the surface activity of PEGylated NPs has shown that the presence of PEG as a surface coating material significantly decreases interfacial tension (or increases surface pressure). Björkegren et al.³³ have analyzed the surface activity of PEG-functionalized silica NPs at air/water interface and observed that NPs surface activity is proportional to the degree of NP surface functionalization. Here, we

employed dynamic surface pressure measurements to evaluate the kinetics of Ag-PEG adsorption at air/water interfaces, the degree of monolayers coverage, and how the presence of lipid monolayers changes these properties. Subphase silver (Ag) and phosphorus (P) concentrations analysis were used to confirm Ag-PEG adsorption at interface and the extent of lipid extraction.

EXPERIMENTAL

Materials. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC; zwitterionic lipid) and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG; anionic lipid) were purchased from Avanti Polar Lipids (Alabaster, AL). Fig. 3-1A shows the chemical structure of the lipids. Chloroform (CHCl_3 , >99.8%) was purchased from Fisher Scientific (Waltham, MA). Ag-PEG NPs dispersed in deionized (DI) water were purchased from Ocean NanoTech (San Diego, CA) and used as-received (Fig. 3-1B). Ag-PEG were prepared by the manufacturer by coating silver NPs with organic layers consist of a monolayer of dodecanethiol, a monolayer of anionic amphiphilic polymer covalently modified with PEG. Nitric acid (65-71%, TraceSELECT Ultra grad), standard silver (Ag) solution (1000 mg Ag L^{-1} in nitric acid, TraceCERT[®] grade) and standard phosphorus (P) solution (1000 mg P L^{-1} in H_2O , TraceCERT[®] grade) were purchased from Sigma Aldrich. Deionized (DI) ultra-filtered water for all reported measurements was obtained from a Millipore Direct-Q3 UV purification system (Billerica, MA) at 18.2 $\text{m}\Omega$ resistance and pH 6.5. All materials were utilized as received.

Nanoparticle Characterization. NPs were characterized using transmission electron microscopy (JEOL JEM-2100F) operating at 200 kV and Malvern Zetasizer Nano ZSX for their core radius, and hydrodynamic radius and zeta potentials, respectively. The average core radius (r_c) of Ag-PEG was determined by analyzing multiple TEM images with the ImageJ software ($n > 50$).³⁵ To measure the average zeta potentials (ζ) and hydrodynamic radius (r_h) of Ag-PEG, the as-received particles were diluted ten-fold in deionized water and analyzed at 25 °C. The values reported are based on triplicate measurements of three different samples.

Figure 3-1 (A) Chemical structure of DOPC and DOPG and (B) schematic of Ag-PEG nanoparticles (not to scale).

Monolayer surface pressure measurements. Surface pressures measurements were conducted using a temperature-controlled Langmuir-Blodgett Teflon trough

(model 102M, KSV NIMA, Biolin Scientific Inc., Linthicum Heights, MD) equipped with two symmetrically moving barriers and a paper Wilhelmy plate (KN005, KSV NIMA, Biolin Scientific Inc., Linthicum Heights, MD) as a surface pressure sensor. The trough had a fully opened area of $\sim 80 \text{ cm}^2$ and a width of 7 cm (Fig. 3-2).

Figure 3-2 Schematic of the Langmuir trough system equipped with two symmetrically moving barriers, recirculation tubing and a paper Wilhelmy plate as a surface pressure sensor. The surface pressure measurements were conducted in the presence or absence of lipid monolayers and different bulk concentrations of nanoparticles.

The experiments were conducted at 25 °C through the following steps. (1) The trough and barriers were cleaned thoroughly with chloroform and then ethanol, followed by rinsing with DI water. (2) The trough was then filled with DI water and the Wilhelmy plate was equilibrated in the subphase. (3) The water surface was cleaned through compression/aspiration/expansion cycles and followed by spreading an aliquot of dissolved lipid (DOPC/DOPG; 1:1 mole ratio) in chloroform (1 mM) on the air/water interface. The water subphase volume within the trough was 140 mL and approximately 9×10^{15} lipid molecules were spread at the air/water interface. (4) The solvent was

allowed to evaporate for 45 min and the monolayers were compressed and expanded at a constant barriers rate $10 \text{ cm}^2 \text{ min}^{-1}$ to obtain the surface pressure-area (π - A) isotherms. (5) After recording compression/expansion isotherms, Ag-PEG was injected into the subphase without disturbing the monolayer and dynamic changes in surface pressure ($\Delta\pi$) were monitored for 160 min. (6) The monolayers were then subjected to an additional compression/expansion cycle. The dynamic changes in surface pressure ($\Delta\pi$ - t) and surface pressure-area (π - A) isotherms were recorded at different amounts of Ag-PEG loaded in the subphase (5, 25, 50, 100, 300, and 500 μL).

The compression/expansion rate applied for all runs was $10 \text{ cm}^2 \text{ min}^{-1}$, which corresponded to an area deformation rate, $d(\Delta A/A_0)/dt$, of about $2 \times 10^{-3} \text{ s}^{-1}$. The total area of the trough during the cycles ranged roughly from 20–70 cm^2 . In all isotherm experiments, at least three consecutive cycles were performed and the ones in which the shape of the (π - A) curves remained constant were analysed and are presented here. Ag-PEG were mixed within the subphase by recycling the solution using a peristaltic pump at a flow rate of 0.8 mL s^{-1} . The same monolayer experiments were conducted as mentioned above but in the absence of lipid monolayers to determine the surface activity and adsorption kinetics of Ag-PEG alone. All experiments were conducted at least in duplicate. Sample volumes of 2 mL were removed from the Langmuir trough subphase at the end of monolayer experiments for analysis of silver (Ag) and phosphorus (P) concentrations in the subphase.

NPs (Ag) and lipid (phosphorus) subphase concentration analysis.

Ultraviolet–visible spectroscopy (UV–vis, model: Cary 50, Varian, Palo Alto, CA) and inductively coupled plasma mass spectroscopy (ICP-MS, model: iCAP Q, Thermo

Scientific, Waltham, MA) were used to measure the subphase concentrations of Ag and P, respectively. For [Ag] determined by UV-vis spectroscopy, plasmon resonance absorption was measured based on the maximum peak height at wavelengths of 425 nm after baseline subtraction. For [P] determined by ICP-MS, samples were digested using nitric acid (200 μ L) and then diluted 10-fold with DI water. Standard solutions containing different concentration (0.1, 1, 10, 100 and 1000 μ g L⁻¹) of phosphorus were used for instrument calibration. Trace amounts of P measured in deionized water and digestion acid solution were subtracted from the reported values. All measurements were conducted in triplicate.

RESULTS AND DISCUSSION

Ag-PEG characterization. Ag-PEG nanoparticles were characterized prior to the monolayer experiments for their size, zeta potentials, stability and extent of dissolution. As shown in Fig. 3-3A, the average core radius (r_c) was 6 ± 2 nm based on analysis of TEM images. The polymer coatings surrounding Ag-PEG were not observed in the micrographs. The mean hydrodynamic radius (r_h) and zeta potential (ζ) were measured to be 15 ± 2 nm (0.04 polydispersity index) and -10.6 ± 0.1 mV, respectively. The average coating thickness based on the difference between r_h and r_c was 9 nm. The maximum surface plasmon resonance (SPR) absorbance was observed at a wavelength of 425 nm (Fig. 3-3B). Ag-PEG SPR absorbance was measured by UV-vis spectroscopy over 3 months, and there was no significant shift and reduction in the SPR wavelength indicating that the nanoparticles were stable. Similar to our previous study on anionic (COOH) and cationic (NH)-coated AgNPs,²⁶ considering that the monolayer

experiments were conducted within 3 months of receiving the samples, we did not account for NP dissolution in our analysis.

Figure 3-3 (A) Histogram plot of Ag-PEG NPs core radius, r_c , based on TEM analysis (inset: representative micrograph). The core radius of the NPs was determined by analyzing TEM images with the ImageJ software ($n > 50$); (B) UV-vis spectra of Ag-PEG NPs over 3 months.

Dynamic surface pressure measurements. The adsorption of Ag-PEG was first examined at air/water and air/lipid/water interfaces at Ag-PEG concentrations from 0.04 to 3.55 mg L⁻¹. Dynamic changes in surface pressure, $\Delta\pi$, were determined as $\Delta\pi = \pi(t) - \pi_i$, where $\pi(t)$ is the dynamic surface pressure after NPs injection and π_i is the initial surface pressure of the air/water ($\pi_i = 0$ where $\gamma_i = \gamma_0 \approx 70$ mN m⁻¹; Fig. 3-4A) or air/lipid/water ($\pi_i = 10$ where $\gamma_i = \gamma_L \approx 60$ mN m⁻¹; Fig. 3-4B) interfaces.

As shown in Fig. 3-4C, Ag-PEG were surface active as observed by the increase in surface pressure ($\Delta\pi$) over 160 min with increasing Ag-PEG concentration. The surface activity can be attributed to the PEGylated amphiphilic polymer coating. At high concentrations ($[\text{Ag-PEG}] \geq 0.71 \text{ mg L}^{-1}$), the long-term gradual increase in $\Delta\pi$ after the initial adsorption (up to 30 min) suggests that there may have been an adsorption barrier that limited Ag-PEG adsorption. This barrier may have been due to high surface pressures, or fewer ‘vacant sites’ available for Ag-PEG adsorption, where Ag-PEG diffused back into the bulk phase and increased the timescale of the dynamic surface pressure changes.^{36,37} Björkegren et al. have reported similar results for PEGylated silica NPs surface activity at air/water interface.³³

In the presence of DOPC/DOPG monolayers at an initial surface pressure of 10 mN m⁻¹ (Fig. 3-4B) Ag-PEG remained surface activity and the lipid monolayer did not prevent Ag-PEG adsorption at the interface (Fig. 3-4D). Considering that both Ag-PEG and DOPC/DOPG monolayers exhibit a net negative charge, adsorption can be attributed to hydrophobic interactions. Xi et al.²¹ have also demonstrated that Ag-PEG similar to those used in this study bind to DOPC/DOPG bilayer vesicles. In their work, it was proposed that the surface activity of the PEG-polymer coating may have facilitated membrane penetration through hydrophobic interactions despite electrostatic repulsion.

Figure 3-4 Schematic of Ag-PEG adsorption at (A) air/water interface ($\pi_i = 0$ where $\gamma_i = \gamma_0 \approx 70 \text{ mN m}^{-1}$) and (B) air/lipid/water interfaces ($\pi_i = 10$ where $\gamma_i = \gamma_L \approx 60 \text{ mN m}^{-1}$). Dynamic changes in surface pressure ($\Delta\pi - t$) are shown after Ag-PEG injection in (C) the absence and (D) the presence of DOPC/DOPG monolayers. (E) Excess Ag-PEG surface concentrations (Γ , NP m^2 or mol m^2) as a function of the equilibrium Ag-PEG concentration and (F) the resulting effective interface radius ($r_{i,\text{eff}}$) of Ag-PEG calculated assuming 2D hexagonal packing.

The dynamic surface pressure measurements show that Ag-PEG are surface active in the absence and presence of the lipid monolayer. However, greater surface activity was observed at the air/water interface, which suggests that the presence of a lipid monolayer may have reduced Ag-PEG adsorption or that Ag-PEG may have removed lipids from the interface. To address this, the subphase concentrations of Ag-PEG and phosphorus [P] were analyzed by UV-vis spectroscopy and ICP-MS, respectively. In the case of Ag-PEG, the excess surface concentration, Γ , was determined by mass balance as $\Gamma = (c_i - c_{eq})_b V(V_{NP}\rho_{Ag}A)^{-1}$ where $(c_i - c_{eq})_b$ is the change in bulk Ag-PEG concentration from initial (c_i) to pseudo-equilibrium (c_{eq}), V_{NP} is the mean Ag-PEG volume based on r_c , ρ_{Ag} is the density of silver, and V and A are the trough volume and area, respectively. The maximum surface concentration, Γ_∞ , was determined as $\Gamma_\infty = 0.9069A_{NP}^{-1}$ where 0.9069 is the 2D hexagonal packing density of spheres and A_{NP} is the cross-sectional area of Ag-PEG based on the mean hydrodynamic radius, r_h . The effective interface radius of the nanoparticles ($r_{i,eff}$) were also calculated based on excess Ag-PEG surface concentration and assuming 2D hexagonal packing of spherical NPs (Fig. 3-4F).

Excess Ag-PEG surface concentrations are shown in Fig. 3-4E as a function of the equilibrium Ag-PEG concentration, $[Ag-PEG]_{eq} = c_{eq}$. Based on Γ , greater Ag-PEG adsorption was observed in the absence of a lipid monolayer. The reduction in Ag-PEG adsorption when a lipid monolayer was present ranged from 21 to 33% at high Ag-PEG concentrations ($\geq 0.71 \text{ mg L}^{-1}$). However, the extent of Ag-PEG adsorption in the presence of a lipid monolayer suggests that Ag-PEG did not simply displace the lipids from the interface or that the lipids packed more tightly at the interface to accommodate

Ag-PEG (which would have led to a significant increase in surface pressure). This was confirmed by the subphase phosphorous concentration (each lipid molecule contains a single P atom). Values for [P] in the bulk in the absence and presence of a lipid monolayer were similar, suggesting that lipid extraction was not a significant factor (Fig. 3-5). Therefore, we conclude that Ag-PEG did not extract lipids from the monolayers and that the lipids remained at the interface to form a mixed Ag-PEG + lipid film.

Figure 3-5 Subphase phosphorus concentration determined by ICP-MS (note that each lipid molecule contains a single P atom in the headgroup). Error bars represent standard deviation from average value. P total reflects the total amount of lipid added to the air/water interface.

Monolayer $\pi - A$ isotherms. Results from dynamic surface pressure show that Ag-PEG and lipid + Ag-PEG monolayers form at low surface pressures. Surface pressure-area ($\pi - A$) isotherms were measured to determine the stability of these films and, in the case of lipid + Ag-PEG monolayers, to determine if the ability to lower

interfacial tension (or raise π) is additive. Isotherms for Ag-PEG, lipid, and Ag-PEG + lipid are shown in Fig. 3-6. For Ag-PEG, a dense monolayer was formed at $[\text{Ag-PEG}] \geq 0.71 \text{ mg L}^{-1}$ as demonstrated by the high surface pressure (up to 45 mN m^{-1} upon compression), monolayer collapse³⁸, and hysteresis³⁹ upon expansion. These features were not observed at lower $[\text{Ag-PEG}]$, suggesting that there was no aggregation or entanglement at the interface and Ag-PEG formed stable monolayers with reversible compression behavior.

Figure 3-6 A comparison between compression–expansion isotherms of Ag-PEG NPs at air/water and air/lipid/water interface, at Ag-PEG concentrations from 0.04 to 3.55 mg L^{-1} (the compression is the higher curve, and the expansion is the lower curve in each case).

The collapse pressure (π_c , mN m^{-1}) and collapse area (A_c , cm^2) were determined from $\pi - A$ isotherms of Ag-PEG at high nanoparticle concentrations (0.71 to 3.55 mg L^{-1}) (Fig.3-7). The collapse pressure was directly proportional to Ag-PEG concentration. Based on A_c , and assuming 2D hexagonal packing, an effective Ag-PEG radius of $12.5 \pm 3.9 \text{ nm}$ was calculated at the interface. The calculated ‘interface radius’ of the nanoparticles is consistent with the measured hydrodynamic radius. Hence, Ag-PEG assembled as densely packed monolayers at the air/water interface at high concentrations, and the monolayers collapsed once they exceeded hexagonal packing.

Figure 3-7 The collapse pressure (π_c , mN m^{-1}) and collapse area (A_c , cm^2) from π -A isotherms of Ag-PEG at high nanoparticle concentrations (0.71 to 3.55 mg L^{-1}).

A comparison between compression/expansion isotherms of Ag-PEG at air/water and air/lipid/water interfaces are shown in Fig. 3-6. At low Ag-PEG concentrations ($[\text{Ag-PEG}] \leq 0.35 \text{ mg L}^{-1}$), the isotherm shifted to smaller area with respect to the isotherm of the lipid mixture alone, noting that more compression was necessary for the

Ag-PEG + lipid films to attain the same arbitrary surface pressure compared to pure lipid film. This behavior is not attributed to the extraction of lipid molecules (Fig. 3-5), but rather lipid adsorption onto Ag-PEG at the air/water interface that rendered the Ag-PEG more hydrophobic and reduced the effective lipid surface concentration for lowering interfacial tension (i.e. a ‘subtractive’ effect). At higher Ag-PEG concentrations ($[AgPEG] \geq 0.71 \text{ mg L}^{-1}$) the surface pressure of mixed Ag-PEG + lipid films were greater than the individual components at low surface areas, denoting an ‘additive effect.’ This apparent additive effect was not observed at high surface pressures where the π -A isotherms more closely resembled that for Ag-PEG than for lipids. At these conditions the isotherms were also reversible, noting that upon compression Ag-PEG did not squeeze out or escape into the subphase. Therefore, we concluded that in presence of lipids, there was a composite Ag-PEG + lipid film at the interface, where most of the adsorbed entities remained at the air/water interface.

CONCLUSION

Previous studies have demonstrated that NP-lipid monolayer and lipid bilayer interactions are not only governed by electrostatic interactions, and our work highlights the role of hydrophobic interactions in NP adsorption or penetration into net anionic lipid monolayers, where electrostatic repulsion between anionic NPs and anionic DOPG lipids hinder NP adsorption. Our results show that surface active Ag-PEG can adhere to and perturb net anionic lipid monolayers. Hydrophobic interactions appear to be a main driving force for Ag-PEG adsorption, where the presence of an anionic lipid appears to play a minimal role in reducing Ag-PEG adsorption to the interface. Furthermore, noting that upon compression Ag-PEG do not squeeze out or escape into the subphase, we

conclude that in presence of lipids there is a composite Ag-PEG + lipid film at the interface. In these films, the adsorbed material remains at the interface. Ag-PEG likely cover themselves with lipids in a self-assembly process, thus becoming an integral part of the interfacial film. This finding is in agreement with recent studies where it has been shown that hydrophobic NPs can be encapsulated by a surfactant lipoprotein corona and trapped at the surfactant monolayer upon compression.⁴⁰

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CHAPTER 4

Human Serum Protein Coronas Alter Interactions Between Nanoparticles and a Model Red Blood Cell Membrane

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ABSTRACT

Nanoparticles (NPs) in contact with biological fluids are rapidly covered by a protein corona (PC), composed of hard (strongly bounded) and soft (loosely associated) protein layers. The PC governs the biological ‘identity’ of a NP and represents the actual nano-interface that is presented to and interacts with biological systems. Understanding of such interactions can provide insight into the potential adverse effects associated with the presence of NPs in the environment. Here, we used unmodified, carboxylate-modified and amine-modified polystyrene (PS) NPs to examine the influence of NP surface functional groups on the assemblage of protein corona and its subsequent impact on NP binding to model cell membranes. Lipid monolayers mimicking a red blood cell (RBC) were employed as a model cell membrane and the classical model of Ward and Tordai was applied to quantitatively analyze dynamic surface tension (DST) data. Our results show that bare NPs are not surface-active. They adhere to the monolayer via attractive short-range ion-dipole interactions and induce lipid condensation. HSA corona complexation renders the NPs surface active and promotes their attachment via hydrophobic interactions. NP-HC complexes incorporate into the lipid monolayers, cause lipid condensation and lead to the formation of densely packed HSA+RBC film at the interface, in which NP are an integral part of the mixed film.

INTRODUCTION

The environmental concentration of polymeric particles is constantly increasing due to the significant amount of plastic waste that is being disposed in the oceans and soil.¹⁻³ Recent studies on the size distribution of the plastic debris have shown that millimeter-size plastics can be fragmented to even smaller particles, referred to as

micro- and nano-plastics,⁴⁻⁷ which may pose a significant threat both to the environment and human health.⁶⁻¹⁵ The small size of these particles ($<1\mu\text{m}$) makes them a susceptible of ingestion by organisms that are at the base of the food-chain.¹ The potential adverse effects associated with interactions between these materials and biological systems could be comparable to those observed with engineered nanoparticles (ENPs).¹⁶⁻¹⁸ Toxicological studies conducted in vitro and vivo have demonstrated that polymeric ENPs can translocate across living cells to the lymphatic and/or circulatory system,^{19,20} accumulate in secondary organs,²¹ and impact the immune system and cell health.²²⁻²⁴

NP cellular uptake begins with an initial adhesion of the particle to the cell and subsequent interactions with the lipids and other components of the cell membrane. The interfacial and biophysical forces that modulate this process can be examined using lipid bilayers or monolayers as model cell membranes.²⁵⁻³⁴ Two main advantages of model membranes are that: (1) the lipid composition and structure can be precisely controlled, thereby capturing the essential aspects of the real cell membranes, and (2) the membrane organization and disruption can be measured directly using techniques that are not amenable to living cells.¹⁸ Model membranes have been used extensively to study the adhesion of, and in some cases the resulting disruption caused by both carbon-based and inorganic ENPs.³⁵

In the work discussed below, we have examined the response of human red blood cell model membranes to the adhesion of polystyrene (PS) nanoparticles with a particular emphasis on the effect of NP surface chemistry on this process. Physicochemical properties of NPs, such as size, charge and surface chemistry are the

main factors modulating NP durability and solubility in biological media as well as their biocompatibility and membrane interactions.³⁶

Upon encountering biological fluids (e.g. blood, lymph, cytoplasm, cell culture media) nanoparticles are covered by biomolecules – of which proteins have received the most attention, forming what is described as a “corona”.^{37,38} Recent research has revealed that in many cases it is the biomolecular corona that interacts with biological systems and thereby constitutes a major element of the biological identity of the nanoparticle.^{39–44} In particular, the corona is composed of a tightly, but not completely irreversibly, adsorbed layer of biomolecules (“hard” corona), which is surrounded by a more loosely associated and rapidly exchanging layer of biomolecules (“soft” corona).⁴⁵ The formation of a corona has been reported for several nanoparticles, including polystyrene,⁴⁶ silica,⁴⁷ carbon nanotubes,⁴⁸ silver,³⁹ and gold.⁴⁹ The amount, composition, and orientation of biomolecules present in the corona strongly influence NPs adsorption, distribution, and elimination in biological systems and govern their interactions with cellular membranes.^{50–52} Despite the importance of the biomolecular corona in dominating nanoparticle interactions at biological interfaces, the influence of protein corona formation on nanoparticle behavior at biological membranes has only recently begun to receive considerable attention.⁵³

Within the context of nanoparticle-membrane interactions, some studies have demonstrated enhanced adhesion and uptake for serum incubated NPs in comparison to what was observed for bare nanoparticles,^{54–56} while other studies have shown the opposite – reduced adhesion and uptake after incubation in serum.^{57–62} For instance, Lesniak et al.⁶² have examined the adhesion of polystyrene and silica NPs to the cell

membrane and have shown that the presence of biomolecular corona strongly reduces nanoparticle adhesion (and uptake) by reducing nonspecific interactions between NPs and the cell membrane. On the other hand, Chithrani et al.⁵⁶ have reported a greater uptake for gold NPs in the presence of serum proteins on the NP surface. Detailed investigation into how the physicochemical properties of NPs influence corona formation and, in turn, NP adhesion to cell membrane can provide insights regarding the how these physicochemical properties play a role in NP cellular uptake and subsequent adverse effects.

This study focuses on the kinetics of polystyrene nanoparticle (PS NP) adhesion and the monolayer response using a Langmuir-Blodgett technique combined with fluorescence and Brewster angle microscopy. The objectives of this work were (1) to determine how protein coronas on polystyrene nanoparticles (with different surface chemistries) impact the inherent surface activity of the particles, and (2) to investigate NPs and NP-hard corona complexes interactions with a lipid monolayer mimicking a red blood cell (RBC) membrane and applying theories to reveal mechanistic insight into the interactions.

EXPERIMENTAL SECTION

Materials. All materials were used as received unless otherwise noted. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), egg sphingomyelin (SM), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Liss Rhod PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Figure 4-1A shows the chemical structure of the lipids. Unmodified and carboxylate-modified fluorescent

polystyrene (PS-COOH) NPs were purchased from Polysciences Inc. (Warrington, PA). Amine-modified fluorescent polystyrene (PS-NH) NPs and human serum albumin (HSA, lyophilized powder, essentially fatty acid free) were purchased from Sigma Aldrich. NPs were purified before monolayer experiments by centrifugation and rinsing. Phosphate buffer saline (PBS, 10X) was purchased from Fisher Scientific (Waltham, MA). Chloroform (CHCl_3 , >99.8%), acetone ($\text{C}_3\text{H}_6\text{O}$, >99.5%), and ethanol ($\text{C}_2\text{H}_6\text{O}$, >99.5%) from Fisher Scientific (Waltham, MA) were used as solvents for making stock solutions of the lipids and cleaning the Langmuir trough. Deionized (DI) ultra-filtered water for all reported measurements was obtained from a Millipore Direct-Q3 UV purification system (Billerica, MA) at 18.2 m Ω resistance and pH 6.5.

Composition of model membrane. The model monolayer was composed of major lipid molecules naturally occurring in the outer layer of human erythrocytes;^{63–67} 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and egg sphingomyelin (SM), in a percentage ratio of 44.9%, 12% , and 43.1%, respectively. A small quantity (1 mol%) of rhodamine-conjugated phosphatidylethanolamine (PE) lipid was added to this mixture as a fluorescent probe to label model membrane.

Figure 4-1 (A) Chemical structure and composition of lipids in RBC model membrane; (B) Histogram plot of PS NPs core diameter, d_c , based on TEM analysis (inset: a representative micrograph). The core radius (d_c) of the NPs was determined by analyzing TEM images with the ImageJ software ($n > 50$).

Formation of NP-HC complexes. NP-HC complexes were prepared following the procedure reported for carboxylate-modified PS NPs by Silvio et al.^{68,69} Human serum albumin (HSA) was used as a model protein. Sufficient volumes of NP solutions were added to 1.5 mL microcentrifuge tubes to attain final NP concentrations of 1 mg mL^{-1} . HSA (5% in PBS) was added to the microcentrifuge tubes, and the tubes were incubated at 37 °C for one hour. The tubes were subsequently centrifuged three times (18000 rcf, 4 °C) with a PBS solution wash between each centrifugation step. Finally, the sedimented NPs were re-dispersed in PBS to isolate the NPs and associated complexed proteins.

Characterization of NPs and NP-HC complexes. NPs and NP-HC complexes were characterized using transmission electron microscopy (TEM; JEOL JEM-2100F) operating at 200 kV and a Malvern Zetasizer Nano ZSX for their core radius, and hydrodynamic radius and zeta (ζ) potentials, respectively. The average size of PS NPs was determined by analyzing multiple TEM images with ImageJ software ($n > 50$).⁷⁰ To measure the average ζ -potentials and hydrodynamic diameter (d_h) of NPs, the as-received particles were diluted in PBS and analyzed at 25 °C. The values reported are based on triplicate measurements of three different samples. Adsorption of HSA on PS NPs were visualized by performing negative-staining TEM.⁷¹⁻⁷³ One drop of the diluted

NP-HC solution was placed on a carbon coated grid and blotted with filter paper, after which an adequate amount of 2% uranyl acetate was placed on the grid and was dried thoroughly at room temperature before imaging.

NP-HC complexes were analyzed further using ultraviolet–visible–NIR spectroscopy (Jasco, Tokyo, Japan) and thermogravimetric analysis (TGA; TA Q500, New Castle, DE) for their protein contents. UV-vis spectroscopy was used to quantify the amount of HSA adsorbed on the PS NPs surface by measuring UV absorption at 280 nm and subtracting the unbound HSA present in the supernatant from the adsorption of the initial known amount of HSA. Absorption of PBS was used as the reference. TGA was carried out to determine the amount of HSA adsorbed on the NPs by measuring the weight loss of the NP-HC complexes in the range of 200–550 °C due to protein degradation⁷⁴ and subtracting it from the weight loss of the NP in the same range of temperature. Heating was performed in a platinum crucible under a nitrogen flow (60 mL min⁻¹) at a rate of 10 °C min⁻¹ up to 1000 °C.

Monolayer surface pressure measurements. Monolayers experiments were conducted at 23 °C as previously described.³¹ Monolayers were prepared in Teflon® Langmuir-Blodgett trough (KN2002, KSV NIMA, Biolin Scientific Inc., Linthicum Heights, MD) filled with PBS by spreading dissolved lipids in chloroform at the air-water interface and allowing 45 min for the chloroform to evaporate. Isotherms were generated for a single compression/expansion cycle at a barrier rate of 2 cm² min⁻¹ and π was measured using paper Wilhelmy plates. The total area of the trough during this cycle ranged from roughly 70–240 cm². After recording isotherms, the trough was initially set to maintain a constant surface pressure ($\pi_0 = 30$ mN m⁻¹). Once the

monolayer stabilized and π_0 remained constant, the barrier positions were fixed at the corresponding interfacial area and NPs (or NP-HC complexes) were added to the subphase by injecting them behind the barriers without disrupting the monolayer. To determine the adsorption kinetics of NP and NP-HC at the lipid-water interface, dynamic changes in surface pressure (π) was monitored as soon as NPs were added and for over 600 min for NPs and more than 400 min for NP-HC complexes. The initial NP concentration in the subphase was 10 mg L⁻¹, which was estimated to provide excess surface coverage based on the PS NP cross sectional area at a monolayer surface area of 240 cm².

The same monolayer experiments were conducted as mentioned above but in the absence of lipid monolayers to determine the surface activity and adsorption kinetics of NPs and NP-HC complexes at the air-water interface. All experiments were conducted at least in duplicate.

Sample volumes of 2 mL were removed from the Langmuir trough subphase at the end of monolayer experiments for analysis of PS NPs concentrations in the subphase. PS NPs concentration was measured by UV-vis spectroscopy and based on the maximum peak height at wavelengths of 240 nm after baseline subtraction.

Monolayers visualization. The morphology of monolayers was visualized using fluorescence and Brewster angle microscopy. The Langmuir film was transferred to a plasma cleaned glass slide using a Langmuir-Blodgett (LB) deposition technique at constant surface pressure of 30 mN m⁻¹ and deposition rate of 0.5 mm min⁻¹.⁷⁵ A CytoViva microscope equipped with a Dual Mode Fluorescent Module was used to obtain fluorescent images of the deposited film. Brewster Angle Microscopy (BAM)

was used to enable real-time observation of monolayers at the air-water interface in a Langmuir trough. BAM provides information on homogeneity, phase behaviour and the film morphology by detecting changes in the refractive index of the water surface in the presence of surfactants or surface-active molecules.

RESULTS AND DISCUSSION

NP and NP-HC characterization. NPs and NP-HC complexes were characterized prior to the monolayer experiment for their size, ζ -potential, and extent of protein coverage. The average diameter (d) was 98 ± 9 nm based on TEM analysis. This value corresponds to unmodified, carboxylate- and amine-modified PS NPs (Fig. 4-1B). Unmodified PS NPs had a hydrodynamic diameter, d_h , of 99 ± 2 nm (0.02 PDI) and a ζ -potential, of -45 ± 1 mV. PS-COOH NPs had a $d_h = 103 \pm 2$ nm (0.05 PDI) and a $\zeta = -41 \pm 1$ mV. PS-NH NPs had a $d_h = 104 \pm 1$ nm (0.09 PDI) and a $\zeta = +14 \pm 1$ mV.

Exposure of PS NPs to protein led to changes in their hydrodynamic properties. We incubated the PS NPs in human serum albumin (HSA) solution for 60 min to allow NP-HSA complexes to form and separated these complexes from free and weakly complexed HSA via a series of centrifugation and washing steps comparable to those previously used to operationally define the hard corona on nanoparticles (Fig. 4-2A).^{1,50,76} The changes in ζ -potential and d_h of the particles (Fig. 4-2B, C and D) induced by this procedure provided direct evidence of the complexation of PS NPs by human serum protein. Figure 4-2B shows the increase of PS NPs hydrodynamic diameter upon HSA incubation for concentrations ranging from 0.1 – 600 μ M HSA. A slight increase in d_h at low HSA concentrations followed by a sharp increase of d_h in the range of 10 – 300

μM HSA, which reached a plateau at $d_h = 123$ nm for 300 μM HSA. We infer that HSA concentration of 300 μM is sufficient to saturate the NP surface and form a close-packed monolayer of protein corona.⁷¹ The increase in d_h due to corona formation was about 20 nm and was common to all of them, corresponding to a hydrodynamic-shell thickness of 10 nm (Fig. 4-2C). Negative-staining TEM of NP-HC complexes confirmed that the HSA shell thickness on the NPs was 7 ± 1 nm (Fig. 4-2B, inset). Upon protein complexation, the ζ -potential of the particles became either negative in case of amine-modified PS NPs or less negative for unmodified and carboxylate-modified PS NPs, approaching the value measured for the HSA in PBS (-8.1 ± 0.3 mV) (Fig. 4-2D). These data indicate that NPs form complexes with HSA, and that complexation with proteins occurs regardless of NPs charge and surface functional groups.

Considering the HSA dimensions ($76 \times 76 \times 28 \text{ \AA}^3$)⁷⁷ and the nanoparticle surface area (πd_h^2), we estimated that $1.0 \times 10^3 - 1.5 \times 10^3$ HSA molecules (based on different binding configurations) are required to form a close-packed monolayer of protein corona. We confirmed this using TGA and determined the extent of the protein corona associated with NPs. The number of proteins constructing the HSA monolayer was calculated from the weight loss of the NP-HC complexes in the range of 200 – 550 °C due to protein degradation and subtracting it from the weight loss of the NP in the same range of temperature. We obtained a value of $1.0 \times 10^3 - 1.1 \times 10^3$ HSA per NP at saturation, which is in good agreement with our previous estimation. Figure 4-2E shows the quantification of protein adsorption for each particle. There is a clear correlation between the number of proteins adsorbed and the surface charge of the NPs. The presence of amine groups on the particles implies a positive zeta potential and increases

the protein adsorption. On the other hand, carboxylic acid groups on the particles extend a more hydrophilic nature and reduces protein adsorption. These results are in good agreement with the literatures, showing that nanoparticles with a positive surface charge and more hydrophobic surface adsorb more protein (e.g., BSA molecules) than negatively charged or hydrophilic NPs.^{36,38,78–80}

Figure 4-2 (A) Schematic of NPs used in this study and formation of NP-HC complexes (not to scale); (B) The increase in NP hydrodynamic diameter (d_h) upon adsorption of HSA (inset: representative micrograph of PS-HC complexes); (C) Average hydrodynamic diameters (d_h) and (D) ζ -potential of NPs and NP-HC complexes. Measurements were made in PBS and the reported values are based on triplicate measurements of three different samples; (E) Extent of protein corona associated with

NP-HC complexes (inset: schematic of the dimensions of the HSA as an equilateral triangular prism). Bars represent mean values; error bars correspond to one standard deviation for triplicate experiments.

Dynamic surface pressure measurements at the air-water interface. The adsorption kinetics of NPs and NP-HC complexes were investigated by measuring dynamic surface pressure, π , throughout the adsorption process using a Langmuir-Blodgett (LB) technique. Dynamic changes in interfacial tension were determined as $\gamma - \gamma_0 = \pi_0 - \pi$, where γ is the dynamic surface tension after injection of NPs, and γ_0 and π_0 are the interfacial tension and pressure of the pristine interface ($\pi_0 = 0$ where $\gamma_0 = \gamma_w = 72.5 \text{ mN m}^{-1}$).

Dynamic changes in surface tension (DST) for unmodified, carboxylate-modified, and amine modified PS and PS-HC complexes are depicted in Fig. 4-3A-C, respectively. In general, as NPs diffuse from the bulk and adsorb to the interface, they effectively reduce γ . Early in this process, γ decreases relatively slowly due to the adsorption of single particles to a pristine interface. When the surface concentration of NPs increases, γ drops more rapidly. At long times ($t \rightarrow \infty$), where the interface approaches maximum coverage, the rate of NP surface adsorption decreases due to a steric barrier and γ approaches a plateau reflecting a pseudo-equilibrium condition.

As shown in Fig. 4-3A-C, bare NPs were not inherently surface active. Although Brewster angle microscopy images showed the adherence of particles at the air-water interface (Fig. 4-3D-F), the reduction in interfacial tensions due to their attachment,

$\gamma_{\infty} - \gamma_0$, was less than 1.5 mN m^{-1} for all three NP types, independent of their surface functional group.

HSA corona complexation rendered the NPs surface active due to hydrophobic interactions at the air-water interface, which led to a lower equilibrium surface tension ($\gamma_{\infty, +corona} - \gamma_{\infty, -corona} \approx -7 \text{ mN m}^{-1}$). The maximum reduction in interfacial tension was observed for PS-NH-HC, $\gamma_{\infty} - \gamma_0 = 9.8 \pm 0.3 \text{ mN m}^{-1}$, consistent with the greater number of proteins adsorbed on PS-NH surface.

The morphology and packing of NP-HC Langmuir film at the interface was characterized using fluorescence and Brewster angle microscopy. Results are shown in Fig. 4-3G-I. It is observed that for NP-HC complexes, a dense monolayer is formed at equilibrium time ($t \rightarrow \infty$), in which NPs were an integral part of the interfacial film.

Figure 4-3 Dynamic changes in surface tension for (A) PS, (B) PS-COOH, and (C) PS-NH nanoparticles before and after complexation with human serum albumin (HSA), plotted in a semi-logarithm scale; representative fluorescence microscopy (Scale bars = 20 μ m) and BAM images (Scale bars = 300 μ m) of (D) PS, (E) PS-COOH, and (F) PS-NH NP and (G) PS-HC, (H) PS-COOH-HC, and (I) PS-NH-HC complexes at the air-water interface at equilibrium time ($t \rightarrow \infty$).

Excess NP and NP-HC concentrations at the air-water interface. To further quantify the extent of NPs and NP-HC complexes adsorption at the air-water interface, the subphase concentrations of PS were analyzed by UV-vis spectroscopy. The excess

PS surface concentration, Γ , was determined by mass balance as $\Gamma = (c_i - c_{eq})_b V(V_{NP}\rho_{PS}A)^{-1}$ where $(c_i - c_{eq})_b$ is the change in bulk PS concentration from initial (c_i) to pseudo-equilibrium (c_{eq}), V_{NP} is the mean PS NP volume, ρ_{PS} is the density of polystyrene, and V and A are the trough volume and area, respectively. Results are shown in Fig. 4-4. Based on Γ , while greater adsorption was observed for NP-HC complexes compared to bare NPs, the extent of adsorption for all three types of NP-HC complexes was similar ($\Gamma_{+corona} \approx 15 \times 10^{13} \text{ NP m}^{-2}$). This indicates that the adsorption of NP-HC complexes was due to the proteins on the NP surface. The increase in NP adsorption due to corona complexation was about 45% for PS-NH and PS-COOH, and 62% for unmodified PS, suggesting that NP adsorption was mainly driven by hydrophobic forces and that electrostatic forces played a minimal role.

Figure 4-4 The increase in excess PS surface concentration (Γ , NP m^{-2}) due to corona complexation for unmodified, carboxylate-modified and amine-modified PS NPs at the air-water interface.

Adsorption kinetics at the air-water interface. Dynamic interfacial tension data can be further analyzed using the classical model of Ward and Tordai⁸¹ to quantitatively describe the kinetics of NP adsorption. The following asymptotic equations have been employed to interpret data from the early ($t \rightarrow 0$) and late ($t \rightarrow \infty$) times of nanoparticle adsorption.

At early times (first-stage), an individual NP that is adsorbing to the interface encounters a bare interface. Assuming there is no barrier to adsorption at this stage, the rate of particle diffusion through the bulk is the rate-limiting factor and the diffusion-controlled Ward and Tordai mechanism can be applied.⁸¹ Bizmark et al.⁸² modified the Ward and Tordai model to account for NPs larger than 10 nm with adsorption trapping energy exceeding $10^3 k_B T$:

$$\gamma = \gamma_0 - 2N_A |\Delta E| C_0 \sqrt{\frac{Dt}{\pi}} \quad (1)$$

Here, N_A is Avogadro's number, ΔE is the trapping energy of a single particle at the interface, D is its diffusion coefficient, and C_0 is the molar concentration. The number of NPs adsorbed at the interface is significantly less than that remaining in the bulk and C_0 is assumed to be constant throughout the adsorption process.

Surface coverage at any time during the adsorption process can be calculated from the measured surface tension:⁸²

$$\frac{\theta}{\theta_\infty} = \frac{\gamma_0 - \gamma}{\gamma_0 - \gamma_\infty} \quad (2)$$

where θ_∞ is the maximum fraction of surface coverage, which is 0.91 for hexagonal close packing of spheres⁸³, γ_0 is the pristine interfacial tension of water, and γ_∞ is the

equilibrium interfacial tension. For native NPs, considering that they were not surface active, θ_∞ was determined based on calculated excess PS surface concentrations at the end of adsorption process and was less than 0.5 for all three types of them. We note that for NP-HC complexes, $\theta_\infty = 0.91$ as they were surface active and assembled as densely-packed monolayers at the air-water interface.

Considering the first-stage adsorption energy as $|\Delta E_{est}| = (\gamma_0 - \gamma_\infty)\pi r^2/\theta_\infty$, and the final surface coverage as $\theta_{end1} = 0.3$,⁸³ the effective diffusion coefficient, D_{stg1} , was calculated based on equation (1) from the slope of linear regressions at early time DST data against $t^{0.5}$. Table 1 reports the values of D_{stg1} for NPs and NP-HC complexes and compares them with the diffusion coefficients predicted by Stoke-Einstein equation as $D_{SE} = k_B T / 6\pi\mu r$, in which r is the hydrodynamic radii of the particles and μ is the viscosity of water at room temperature. As summarized in Table 1, the values of D_{stg1} and D_{SE} are within the same order of magnitude, indicating that equation (1) is valid during the early times adsorption of particles from the bulk to the air-water interface.

Using D_{SE} values, we were able to extract the first-stage adsorption energy, $|\Delta E_{stg1}|$, by fitting the slope of early time DST data against $t^{0.5}$. As shown in Table1, there was a clear correlation between the adsorption energy and the ζ -potential of the NPs. Anionic unmodified and carboxylate-modified PS had similar adsorption energy, while greater values were observed for cationic amine-modified PS. Anionic PS NPs were electrostatically repelled from the interface, since the ζ -potential at the air-water interface has been shown to be negative.^{84,85}

Table 4-1 Calculated Stoke-Einstein diffusion coefficient (D_{SE}); estimated adsorption energy ($|\Delta E_{est}|$); computed early time effective diffusion coefficient (D_{stg1}), and adsorption energies ($|\Delta E_{stg1}|$ and $|\Delta E_{stg2}|$); calculated long-time adsorption constant (k_a). Errors correspond to one standard deviation for triplicate experiments.

			Stage 1 (Diffusion-controlled)		Stage 2 (Denaturation)	Stage 3 (Barrier-controlled)
	$D_{SE}(\times 10^{-12})$ ($m^2 s^{-1}$)	$ \Delta E_{est} (\times 10^4 k_B T)$ (J)	$D_{stg1}(\times 10^{-12})$ ($m^2 s^{-1}$)	$ \Delta E_{stg1} (\times 10^4 k_B T)$ (J)	$\Delta E_{stg2}/\Delta E_{stg1}$	$k_a(\times 10^{-6})$ ($m s^{-1}$)
PS	4.91 ± 0.12	1.41 ± 0.13	7.92 ± 0.78	1.35 ± 0.02	—	—
PS-COOH	4.73 ± 0.29	1.46 ± 0.57	6.78 ± 0.15	1.81 ± 0.05	—	—
PSNH	4.63 ± 0.35	1.44 ± 0.25	6.25 ± 0.18	2.61 ± 0.02	—	—
PS-HC	3.99 ± 0.30	2.57 ± 0.43	3.78 ± 0.37	8.11 ± 0.19	6.86	1.70 ± 0.19
PS-COOH-HC	3.71 ± 0.27	2.96 ± 0.45	3.31 ± 0.18	6.13 ± 0.24	7.43	1.51 ± 0.93
PS-NH-HC	3.54 ± 0.16	3.94 ± 0.42	4.54 ± 0.93	8.99 ± 1.03	11.39	5.83 ± 0.42

In the first-stage approximation ($\theta < 0.3$) proposed by Bizmark et al.,⁸² only one slope was observed when DST was plotted against $t^{0.5}$. We observed similar behavior for NP adsorption at early times. However, for NP-HC complexes, two distinct stages with clearly different slopes were noted in a plot of early time DST over $t^{0.5}$ (Fig. 4-5B) consistent with the results of recent work by Tian et al.⁸⁶ using poly(ethylene oxide) (PEO)-modified polystyrene NPs to study the adsorption kinetics at the air-water interface.⁸⁶ The presence of two distinct stages at the early time adsorption were comparable when NP-HC complexes were employed. As shown in Fig. 4-3, although the transition between two stages occurs at an earlier time for PS-NH-HC compared to PS-HC and PS-COOH-HC, no statistically significant difference in interfacial tension is observed at the transition point for all three types of NP-HC complexes. For the first-stage, we calculated the adsorption energy of NP-HC complexes using the slope of the

DST over $t^{0.5}$ in equation (1). As listed in table 1, PS-COOH-HC complexes have smaller adsorption energy compared to unmodified and amine-modified PS-HC consistent with the lower protein content calculated for carboxylate-modified PS. The relation between the extent of protein corona associated with NPs and their first-stage adsorption energy highlights the role of hydrophobic interactions as a main driving force for NP-HC complexes adsorption at early times.

For the second stage, a much larger slope in the plot of DST over $t^{0.5}$ was observed. We calculated the ending surface coverage of stage 1, θ_{end1} , based on the measured final surface tension, γ_{end1} , of the first linear regime when $\gamma - \gamma_0$ was plotted against $t^{0.5}$. For all three NPs, θ_{end1} was similar and less than 0.05. Hence, we inferred that NP adsorption during stage 2 is diffusion-controlled and the Stokes–Einstein equation can be applied to estimate the diffusion coefficient of NPs. The new adsorption energy $|\Delta E_{estg2}|$ is then calculated by fitting the second stage slope of surface tension over $t^{0.5}$ (Table 1). The observed two-stage transition for NP-HC complexes is attributed to protein denaturation at an interface.⁸¹ HSA has hydrophilic groups on its surface that make it water-soluble, but hydrophobic peptide residues in the core. Proteins denature at a hydrophobic interface wherein the hydrophobic core peptides unfold at the interface, while the hydrophilic peptides orient toward the aqueous phase. The extent of increase in adsorption energy due to HSA denaturation at the air-water interface was consistent with the extent of HSA associated with NPs. The greater increase in $|\Delta E|$ was observed for PS-NH-HC, while unmodified and carboxylate-modified PS NPs showed similar values.

Figure 4-5 (A) Dynamic changes in surface tension over time for PS-COOH-HC complexes, where three stages of behaviour are displayed; duplicate experiments depicting changes in DST (B) over $t^{0.5}$, at early times where the adsorption is diffusion-controlled; and (C) over $t^{-0.5}$, during the later stage of adsorption when it is barrier-controlled. Linear fits are observed. Points represent experimental data, and solid lines represents the observed trend.

During the later stage of adsorption ($t \rightarrow \infty$ and $\theta > 0.75$)⁸², as the interface approaches the maximum coverage, the presence of already adsorbed particles hinders the attachment of adsorbing particles. The later stage adsorption kinetics can be described by introducing a blocking function to the long-time Ward and Tordai approximation to account for the adsorption barrier at high NP surface coverage:⁸⁷

$$\gamma = \gamma_{\infty} + \frac{K_1 |\Delta E|}{(\pi r^2)^2 N_A C_0} \sqrt{\frac{1}{Dt}} \quad (3)$$

$$K_1 = \theta_{\infty} \sqrt{\frac{\theta_{\infty}}{4.64 k_a}} \quad (4)$$

where, K_1 is the dimensionless reaction coefficient, and $\overline{k_a}$ is the dimensionless adsorption constant. The adsorption constant, k_a , can be determined as, $k_a = \overline{k_a} D N_A C_0 \pi r^2$.

For native PS NPs, the maximum surface coverage for native NPs, θ_∞ , was less than 0.75, indicating that adsorbing particles never experience a crowded interface and the adsorption is diffusion-controlled at any time during the process. For NP-HC complexes, as shown in Fig. 4-4C for PS-COOH-HC, we calculate the adsorption constant, k_a , from the gradient of DST over $t^{-0.5}$ at the later stage (Fig. 4-5C). The values of k_a for all NPs are listed in Table 1. Clearly, PS-NH-HC complexes have a greater adsorption constant compared to unmodified and carboxylate-modified PS-HC complexes. The greater value of k_a for PS-NH-HC denotes a faster rate of adsorption in stage 3, which is consistent with the greater adsorption energy and the protein content calculated for amine-modified PS compared to unmodified and carboxylate-modified PS.

RBC monolayer morphology and π -A isotherm. Surface pressure-area isotherm of the mixed lipid film mimicking the outer leaflet of human RBC membrane is shown in Fig. 4-6A. Increasing π corresponded to a decrease in A with compression as the lipids packed more tightly at the interface. There was a continuous phase transition from the gaseous (G)-phases at large lipid molecular area ($A \approx 113 \text{ \AA}^2 \text{ molecule}^{-1}$) to coexisting liquid-expanded (LE)-liquid-condensed (LC) phases at lower lipid molecular area where $\pi \geq 15 \text{ mN m}^{-1}$, with the monolayer collapse occurring at $\pi \approx 43 \text{ mN m}^{-1}$. The morphology of the film was visualized *in situ* using Brewster angle microscopy (BAM) technique. The BAM images were taken throughout the monolayer

compression isotherm at 23 °C. The structure of lipid domains was further characterized using fluorescence microscopy. Rhodamine conjugated mono-unsaturated phosphatidylethanolamine (PE) lipid was used as a fluorescent probe. Labeled Langmuir monolayers at specific constant π (10, 15 and 30 mN m⁻¹), were transferred to a plasma cleaned glass slide performing a Langmuir-Blodgett (LB) deposition technique and imaged after drying out at room temperature. Representative fluorescence and Brewster angle microscopy images of the monolayers are shown in Figure 6B. The morphology of the films is comparable to that reported previously in the literature for the same lipid system.^{63–65} RBC monolayers existed as G phases at $\pi = 0.1$ mN m⁻¹ and mixed of two LE phases at $\pi = 10$ mN m⁻¹. First domains of LC phases appeared at $\pi = 15$ mN m⁻¹. These domains existed up to the collapse point, while they enlarged with further compression and took a flower-like characteristic of pure SM monolayer.

Figure 4-6 (A) surface pressure-area (π - A) isotherm of the monolayer at the air/water interface at 23 °C; (B) representative fluorescence microscopy (Scale bars = 20 μm) and BAM images (Scale bars = 300 μm) of the film during a compression isotherm.

Dynamic surface pressure measurements at the air-lipid-water interface. The adsorption kinetics of NPs and NP-HC complexes were then examined at the air-lipid-water interface by measuring dynamic changes in RBC monolayer surface pressure throughout the NP adsorption process. The dynamic changes in interfacial tension were determined as $\gamma - \gamma_0 = \pi_0 - \pi$, here γ_0 and π_0 are the interfacial tension and pressure of the air-lipid-water interface ($\pi_0 = 30$ where $\gamma_0 = \gamma_L = 42.5 \text{ mN m}^{-1}$).

Dynamic changes in RBC monolayer surface tension due to the adsorption of unmodified, carboxylate-modified, and amine modified PS NPs and NP-HC complexes are shown in Fig. 4-7A-C, respectively. The monolayer responded similarly to the adsorption of all three NP types, displaying an increase in interfacial tension. While the extent of increase in γ was almost equivalent for unmodified and amine-modified PS ($\gamma_\infty - \gamma_0 \approx 22 \text{ mN m}^{-1}$), less increase was observed for carboxylate-modified PS ($\gamma_\infty - \gamma_0 \approx 18 \text{ mN m}^{-1}$). The increases in interfacial tension suggest that PS NPs did not penetrate the monolayer, but rather remained bound to the monolayer below the interface and caused lipid condensation (i.e. a reduction in the effective area per lipid). It has been shown that anionic nanoparticles can bind to zwitterionic lipid monolayers and bilayers through attractive interactions with the positive group of zwitterionic lipids (e.g. choline group of POPC and ethanolamine group of POPE).⁸⁸⁻⁹⁰ Moreover, zwitterionic lipids have dipole moments extending into the aqueous phase that can lead

to attractive short-range ion-dipole interactions. Both anionic and cationic nanoparticles can reorient the headgroup dipoles of zwitterionic lipids, causing the dipole to orient perpendicularly to the air-water interface and reducing the area per lipid.⁹⁰ Hence, lipid condensation in the RBC monolayers can be attributed to the dipoles reorientation of zwitterionic POPC and POPE. We observed similar behaviour in our previous work using carboxylate- and amine-modified silver NPs and PC/PG monolayers.²⁵

The morphology of the monolayer was visualized *in situ* using Brewster angle microscopy (BAM) technique. As shown in Fig. 4-8A, the extent of lipid condensation was greater for PS-NH compared to unmodified PS and PS-COOH, suggesting that inclusion of cationic nanoparticles within a monolayer induces more modification in the monolayer lipid packing.

Figure 4- 7 Dynamic changes in surface tension for (A) PS, (B) PS-COOH, and (C) PS-NH nanoparticles before and after complexation with human serum albumin (HSA), plotted in a semi-logarithm scale.

The extent of increase in RBC monolayer DST due to the adsorption of NP-HC complexes was smaller compared to that for bare NPs (Fig. 4-7), indicating that NP-HC complexes induced less lipid condensation. These results are consistent with our previous work using cationic and anionic silver nanoparticles and show that hydrophobic interactions were responsible for NP insertion, while electrostatic and charge-dipole interactions were responsible for lipid condensation. Moreover, real time BAM imaging of the film displayed lipid condensations at early time NP-HC complexes adsorption, and the formation of homogenous densely packed monolayer at equilibrium (Fig. 4-8B1&2). Hence, we infer that at early times, NP-HC complexes penetrate into monolayers through attractive short-range ion-dipole interactions, bind to zwitterionic lipids and cause lipid condensation (increasing γ) similar to what we observed for bare NPs adsorption. This process follows by the protein corona partitioning between coexisting membrane domains via attractive hydrophobic interactions (increasing γ) and unfolding at the air-water interface.^{91, 92} This leads to the formation of homogenous densely packed RBC+HSA film at the interface, in which NPs are an integral part of the mixed film.⁹³ This behaviour was common to all three NP-HC complexes.

Figure 4-8 BAM images of the RBC monolayer response to (A) NPs adsorption at equilibrium ($t \rightarrow \infty$), (B1) NP-HC complexes adsorption at early time, and (B2) NP-HC complexes adsorption at equilibrium ($t \rightarrow \infty$). Scale bars = 300 μm .

Excess NP and NP-HC concentrations at the air-lipid-water interface. To further quantify the extent of NP and NP-HC complex adsorption at the air-lipid-water interface, the subphase concentrations of PS were analyzed by UV-vis spectroscopy. Γ_{+RBC} was determined by mass balance as explained earlier. Results are shown in Fig. 4-9. In general, the presence of a lipid monolayer led to a decrease in the excess surface concentration of all three NPs and NP-HC complexes, suggesting that the RBC monolayer acts as a steric barrier at the interface and hinders the attachment of adsorbing

particles. The reduction in excess concentration was about 80% for unmodified PS and PS-COOH, and 50% for PS-NH. There was a clear correlation between the excess NP concentration at the air-lipid-water interface (Γ_{+RBC}), and their adsorption energy, $|\Delta E_{stg1}|$. Unmodified PS and PS-COOH had similar $|\Delta E_{stg1}|$ and Γ_{+RBC} , while greater values were observed for PS-NH. We infer that the adhesion of PH-NH NPs to the interface was more favorable simply due to their greater $|\Delta E_{stg1}|$.

Higher Γ_{+RBC} was observed for NP-HC complexes compared to bare NPs, indicating that NP-HC complexes were able to overcome the steric barrier and attach to the interface. This can be attributed to the presence of HSA corona on the NP surface which renders the particles surface active. The reduction in Γ due to the presence of RBC monolayer was about 20% for unmodified PS-HC and PS-COOH-HC, and less than 4% for PS-NH-HC, consistent with the greater number of proteins adsorbed on PS-NH NPs surface which led to the higher $|\Delta E_{stg2}|$ and k_a .

Figure 4-9 The ratio of excess PS surface concentration at the lipid-water interface (Γ_{+RBC} , NP m⁻²) to the values at the air-water interface (Γ_{-RBC} , NP m⁻²) for unmodified, carboxylate-modified and amine-modified PS NP before and after HSA complexation.

CONCLUSIONS

In this work, we coupled dynamic surface pressure measurements with fluorescence and Brewster angle microscopy to investigate how the NP interfacial activity and cell membrane interactions are modulated by the presence of a protein corona, and to explore its effect on the morphology and structure of cell membrane lipid domains. Dynamic interfacial tension data were further analyzed using the classical model of Ward and Tordai to quantitatively describe the kinetics of NP adsorption and to identify the key parameters that control the duration and extent of nanoparticle binding and the monolayer response. Our results show that although bare NPs were not surface-active, they could adhere to the monolayer via attractive short-range ion-dipole interactions and induce lipid condensation. HSA corona complexation rendered the NPs surface active and promoted their attachment via hydrophobic interactions. For NP-HC complexes, there was a clear correlation between the extent of HSA associated with NPs and their interfacial activity such as time scale and extent of adsorption, and adsorption energy and constant. When RBCs were present, NP-HC complexes incorporated into the lipid monolayers, induced lipid condensation and led to the formation of densely packed HSA+RBC film at interface, in which NPs were an integral part of the film. This study represents an initial demonstration of the interfacial interactions occur when nanoparticle-HSA corona complexes interact with model cell membranes. Whereas more studies are necessary to generalize these results, other studies with serum corona have shown similar features.^{50,94} Thus, the results presented here are expected to be most transferable to nanoparticles biomolecular corona.

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CHAPTER 5

CONCLUSIONS AND FUTURE WORK

In this work, we have examined the response of model cell membranes to the adhesion of nanoparticles with a particular emphasis on the effect of NP charge, surface functional groups and interfacial activity on this process. Our results highlight the role of interfacial interactions, notably electrostatic, hydrophobic, and van der Waals interactions, in governing the timescale for NP-cell membrane association, membrane disruption, and the extent of NP adhesion.

Anionic AgNPs inserted into both saturated and mono-unsaturated net anionic monolayers at a low initial surface pressure (10 mN m^{-1}) and caused lipid condensation at high initial surface pressures (20 and 30 mN m^{-1}). Hydrophobic interactions were responsible for insertion, while electrostatic and charge-dipole interactions with zwitterionic lipids were responsible for condensation. Cationic AgNPs inserted only into saturated monolayers and otherwise led to lipid condensation. For Ag-NH, adsorption was driven primarily by electrostatic interactions with anionic lipids in monolayers. Surface-active AgNPs adhered to and perturb net anionic lipid monolayers. Hydrophobic interactions appeared to be a main driving force for Ag-PEG adsorption, where the presence of an anionic lipid appeared to play a minimal role in reducing Ag-PEG adsorption to the interface. Moreover, a higher degree of binding was observed for cationic AgNPs compared to anionic AgNPs, suggesting that NP adsorption was primarily driven by lipid headgroup interactions while the monolayer response was

driven by the lipid tail saturation and phase behavior. Surface excess concentration analysis suggested that surface-active AgNPs assembled as densely-packed monolayers in the presence and absence of a lipid monolayer. The presence of a lipid monolayer led to only a slight decrease in the excess surface concentration of AgPEG NPs. Inclusion of PS nanoparticles within model RBC monolayers modified the monolayer lipid packing and induced local lipid condensation. Complexation of PS NPs with proteins promoted their attachment to the lipid monolayers. PS NP-hard corona (HC) complexes caused lipid condensation and led to the formation of densely packed HSA+RBC film at the interface in which NP were an integral part of the interfacial film.

This study contributes to further understanding of the membrane's role in ENP cytotoxicity and cellular uptake and provides insight into the design of biocompatible nanomaterials with minimal or controlled membrane activity. However, current knowledge on the influence of particles in the physiological response of cell membranes is not completely sufficient in providing a general perspective of the problem. This is because in many cases, the conclusions extracted from the experimental results lead to a contradictory picture ascribed to different facts such as NPs concentration in the system, methodologies used to probe the system, or the relevance of the temperature used. Even though the quantification of the real effect of particles on the cell membrane remains challenging, recent research efforts have led to important insights into the effect of the incorporation of particles on the cell membrane properties and the potential toxicological implications of this incorporation on the normal physiological response of the membrane lipids. However, this early success should stimulate research for

developing systematic protocols and methodologies to deepen the understanding of the different aspects involved in this complex health problem.