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Biophysical Characterization of Nanoparticle−Endothelial Model Cell Membrane Interactions

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Abstract: Understanding the biophysical interactions of nanoparticles (NPs) with cell membranes is critical for developing effective nanocarrier systems for drug delivery applications. We developed an endothelial model cell membrane (EMM) using a mixture of lipids and Langmuir balance to study its interaction with NPs. Polystyrene NPs of different surface chemistry and sizes were used as a model nanomaterial, and changes in the membrane's surface pressure (SP) were used as a parameter to monitor its interactions with NPs. Aminated NPs (60 nm) increased SP, plain NPs reduced it, and carboxylated NPs of the same size had no effect. However, smaller NPs (20 nm) increased SP irrespective of surface chemistry, and serum did not influence their SP effect, whereas it masked the effect of larger (>60 nm) plain and carboxylated but not that of aminated NPs. Membranes formed with a single phospholipid showed a different pattern of interactions with NPs than that with EMM, signifying the need of using a mixture of lipids representing the respective cells/tissue of interest for a model membrane. The particular effect of NP characteristics on SP, determined using atomic force microscopy and $\pi-A$ (surface pressure–area) isotherm, can be explained on the basis of whether the interaction results in condensation of phospholipids (increase in SP) or their displacement from the interface into the subphase (decrease in SP), causing destabilization of the membrane. We conclude that NP characteristics significantly influence biophysical interactions with the membrane. Further, the molecular mechanism(s) of nanoparticle interactions with model membranes can be effectively used for optimizing the characteristics of nanomaterials for particular biological applications.

Keywords: Nanomaterials; interfacial properties; Langmuir monolayer; surface pressure; biomimetic model cell membrane

Introduction

Nanoscale carrier systems, such as nanoparticles (NPs),[1,2] block copolymer micelles,[3,4] and liposomes,[5,6] with different surface composition and characteristics are being investigated for their various biomedical applications. Studies have shown

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that the interfacial properties of nanomaterials significantly affect their interactions with the biological environment.[7] For example, a long period of circulation in the bloodstream has been achieved for polymeric NPs and liposomes with surface modifications using poly(ethylene glycol) (PEG) to prevent their opsonization and rapid clearance by the reticuloendothelial system (RES).[8–10] It is also known that surface modifications of nanocarriers with polycationic polymers enhance their cell membrane permeability.[11] Further, the size and hydrophobicity of nanomaterials play important roles in their uptake by the cells of the mononuclear phagocytic system and their eventual clearance by the RES.[8–10,12–14]

While exploring biomedical applications of nanomaterials for *in vivo* use, it is also essential to understand any characteristics of nanomaterials that might cause toxic effects. In a recent study, it has been shown that certain polycationic polymers can create holes in cell membranes, which may result in the leakage of cytoplasmic contents, causing toxicity.[15–17] However, there is very limited information available on the effects of nanocarrier characteristics on interactions with cell membranes. This information can be useful in enhancing the applicability of nanomaterials in the biomedical field, while reducing their toxicity arising from their biophysical interactions with cells and tissues.

The complexity of the processes involved in nanomaterial uptake by cells and the intricacy of the cell membranes make these biophysical interactions more difficult to study and understand in real time. Therefore, biomimetic model cell membranes are used to study such interactions. Among the various model cell membranes, such as supported lipid bilayers[14–16] and liposome membranes,[18] the lipid monolayers using Langmuir films provide a good model system for the biological cell membrane.[19–22] In this model system, lipid molecule composition and the subphase composition and temperature can be set to imitate biological conditions so that the data obtained from this model membrane can be useful in predicting interactions with cell membranes, both *in vitro* and *in vivo*. Generally, in Langmuir model membrane studies, a single phospholipid monolayer of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), which represents the phosphatidylcholine (PC) headgroup, a major component of cell membrane phospholipids, is used.[23] However, a single lipid may not accurately represent others constituting the biological membrane. Only a few studies have reported results from a mixture of phospholipids used as a model membrane.[20–22] Garcia et al.[20] studied the interaction of flurbiprofen sodium with a model membrane that had a phospholipid composition similar to that of corneal cell membrane. Similarly, Egea et al.[21] studied the interaction of cisplatin and cisplatin-loaded NPs with a reticuloendothelial model membrane.

Our aim is to use a biomimetic endothelial cell model membrane (EMM) to delineate the effects of nanomaterial

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characteristics on their interactions with biomembranes. This is important to know because in certain applications it is essential that nanomaterials interact with cell membranes (e.g., gene delivery), whereas in others it is not desirable (e.g., long-circulating nanocarrier systems). Further, nanomaterials are synthesized with different surface chemistry and physical properties for various biomedical applications. However, there is not an easy way to understand how the changes in characteristics of nanomaterials influence interactions with biological membranes and ultimately their intended use. With this aim, we have investigated the effects of NPs with surface chemistry and size on interaction with EMM. We chose polystyrene NPs as a model system because they are available commercially in different sizes and with different surface functional groups. Selected studies were carried out with model membranes formed with a single phospholipid, DPPC or 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), to understand the role of different phospholipids in the membrane on interactions with NPs.

Experimental Section

DPPC, DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS), L-α-phosphatidylinositol (PI), sphingomyelin (SM), and cardiolipin (CL) were purchased from Avanti Polar Lipid (Alabaster, AL). Dulbecco's phosphate buffered saline (D-PBS, obtained from the Central Cell Services' Media Laboratory of our institution) was used as a subphase for monolayer experiments. Polystyrene NPs were purchased from Bangs Laboratories, Inc. (Fishers, IN). Solvents used were of high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Deionized water with 18.2 MΩ·cm resistivity collected from Super Q water system (Millipore Corporation, Billerica, MA) was used in all the experiments.

EMM Lipid Solutions. Depending on the solubility of lipids, different solvents were used to prepare lipid solutions. DPPC, PI, SM, and CL were dissolved in HPLC grade $CHCl_3$, whereas DPPE and DPPS were dissolved in a mixture (4:1) of $CHCl_3$ and CH_3OH. The lipid mixture was prepared by mixing the individual lipid solutions according to the following composition: DPPC (56%), DPPE (24%), PI (8.5%), DPPS (4.3%), SM (6.5%), and CL (1.7%). The above phospholipid composition represents the headgroup chemistry of the phospholipids of the native artery's endothelial cell membrane.[24]

EMM Formation. A Langmuir balance (Minimicro 2, KSV Instruments, Helsinki, Finland) was used to form model membranes. The balance is equipped with a Teflon trough, two hydrophilic Delrin barriers, and a microroughened platinum Wilhelmy plate (perimeter 39.24 mm), which was used to measure surface pressure (SP). The whole apparatus was enclosed in a Plexiglas box to avoid contamination from airborne matter and was placed on a granite vibration isolator. The temperature of the Langmuir trough was maintained at 37 °C by a circulating water bath. Before each use, the trough and the barriers were cleaned with Kimwipes (Kimberly Clark, Irving, TX) using chloroform and ethanol, respectively, and then rinsed with water. The surface of the subphase was cleaned before each experiment by aspirating the surface with a water aspirator. The trough was filled with 50 mL of D-PBS. Each experiment was repeated at least three times and the representative data are shown in figures.

To obtain the SP−area ($\pi-A$) isotherm, 4 μL of the lipid mixture was added dropwise (~0.5 μL) on the D-PBS surface in the trough using a Hamilton digital microsyringe. After waiting for 10 min to allow organic solvents to evaporate, the barriers were compressed at the rate of 3 mm/min until the collapse of the membrane. The compression−expansion isotherm was also collected in a manner similar to that described above but with 5 min wait time between compression and expansion cycle. The film was compressed until 30 mN/m SP. Whilhelmy plate calibration for SP was performed by recording and comparing the DPPC and DPPE isotherm data (SP and mmA of the phase transitions and film collapse) with that in the literature. To form EMMs, 10 μL of the lipid mixture was added on the D-PBS surface, and the barriers were compressed until the SP reached 30 mN/m (~85 $Å^2$ mean molecular area [mmA]). In $\pi-A$ experiments, a lesser amount of lipid mixture (4 μL) was used to ensure that the lipid monolayer exists in the gaseous phase prior to compression. A higher amount (10 μL) was used to form the EMM such that the membrane is formed over the maximum surface area of the Langmuir trough to make available more membrane surface area for interaction with NPs.

Zeta Potential (ζ-Potential) of NPs. A 1% stock suspension of NPs was prepared by diluting 10% of the original stock suspension with water which was further diluted (1:50) in water prior to measuring ζ potential using a Brookhaven 90plus Nanoparticle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY).

NP Interaction with EMM. A 1% suspension of each type of NP was prepared by diluting 10% of the original stock suspension with water. Since the original stock suspension was in water, the dilution of NPs was also prepared in water but it is not expected to change the composition of buffer or influence other parameters because its dilution to 50 mL of D-PBS present in trough. A 50 μL aliquot of diluted suspension of NPs was injected over ~30 s using a 50-μL Hamilton digital microsyringe below the EMM through the injection port without causing disturbance to the membrane. The control (without NPs) experiment involved injection of 50 μL water as above. The change in SP was recorded immediately as a function of time at constant mmA for a period of 20 min. To study the effects of FBS on NP interactions with the membrane, a 1% NP suspension of each type of NP was prepared in 10% aqueous

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solution of serum instead of water. The diluted serum acted as a control in these experiments.

Effects of NP Surface Group and Concentration on Interaction with EMM. The initial study was carried out comparing aminated, carboxylated, and plain (without any surface group) polystyrene NPs of 60-nm size. We selected this size because such NPs, with different surface chemistries, were available. To study the effects of NP concentration, a different volume of each type of 60-nm NP was injected into the trough. The concentration of NPs was calculated from the trough volume (50 mL) and the weight of NPs injected. For instance, a 50 μL injection of 1% NP suspension in 50 mL of buffer in the trough gives a concentration of 10 μg/mL.

Effects of NP Surface Group on $\pi-A$ isotherm of Endothelial Lipid Mixture. In these experiments, the lipids were spread on the surface at a lower density than that used for forming the EMM. The lipid mixture (4 μL) was added onto the buffer surface so that the SP of the monolayer remained at ~0 mN/m. A suspension of NPs was injected as described above after allowing 10 min wait time for the evaporation of solvents. The monolayer was then compressed as above until the film collapsed.

Effects of NP Size on Interaction with EMM. To study the effects of NP size, the SP at 20 min for each size of NP from the SP vs time plot was considered and plotted against the size for each type of NP. Twenty minutes was selected for interaction to allow SP to almost reach steady state. The effects of NP size on SP were also studied in the presence of FBS.

Langmuir–Blodgett Membrane Preparation for Atomic Force Microscopy. The EMMs were transferred on a clean glass substrate before and after interaction with NPs for their analysis by atomic force microscopy (AFM). The glass substrate was immersed into the subphase prior to monolayer formation as described above. Following interaction with NPs for 20 min, the EMM was transferred onto the glass substrate by vertical upstroke through the membrane at a constant rate of 3 mm/min. The transfer ratio (ratio of area of lipid monolayer transferred from buffer surface on to glass substrate) of the film was 1, whereas that in the presence of NPs was 1.2.

The transferred films were allowed to dry for at least 24 h in a desiccator at room temperature. The film surface morphology was studied using a Bioscope AFM (Digital Instruments, Santa Barbara, CA) in tapping mode. The silicon cantilevers used were 125 μm long with a resonance frequency of approximately 325 Hz and a tip radius <10 nm. Images were captured with a lateral scan frequency of 0.5–1 Hz and a set-point ratio of 0.98. The acquired images were flattened using a second-order flattening routine in Digital Instruments software.

Results

SP−Area ($\pi-A$) Measurement in the EMM. The shape of the compression isotherm ($\pi-A$) of the phospholipid mixture showed two distinct regions (Figure 1). The EMM showed a gradual increase in SP until the mmA was 100 Å^2, and then a small kink at ~84 Å^2 prior to rapid increase until the membrane collapse occurred at 55 Å^2. The expansion isotherm followed the compression isotherm with a very small hysteresis (Figure 1, inset).

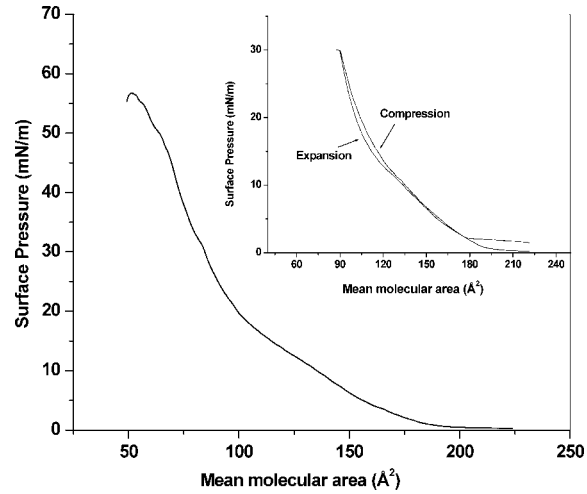

Figure 1. $\pi-A$ isotherm of the endothelial cell model membrane with a lipid mixture consisting of DPPC (56%), DPPE (24%), PI (8.5%), DPPS (4.3%), SM (6.5%), and CL (1.7%). The barriers were compressed at a rate of 3 mm/min until the collapse of the membrane. Inset shows compression−expansion isotherm of the lipid mixture. The wait time between the compression and expansion cycle was 5 min.

Effects of NP Surface Group on Interaction with EMM. NPs with different surface groups interacted differently with the EMM (Figure 2). Aminated NPs showed an initial sharp increase in SP prior to reaching a pseudoplateau at 35 mN/m with time. In contrast, carboxylated NPs increased the SP slightly but then showed a gradual decrease, reaching a SP similar to that of the membrane without NPs. Plain NPs also showed an initial increase in SP but a rapid decline thereafter, ending up below the SP of the membrane without NPs. The magnitude of the initial increase in SP was greater with aminated NPs than with carboxylated or plain NPs.

Effects of NP Concentration on Interaction with EMM. To reflect the effects of concentration of NPs, the change in SP ($\Delta\Pi$) with respect to that of the membrane with water injection at particular time points (2 min 30 s, 10 min, and 15 min) was considered (Figure 3a), and the difference was plotted against the concentration of NPs (Figure 3b). As can be seen from Figure 3b, aminated NPs caused a continuous increase in SP with increasing concentration at all the time points, whereas carboxylated NPs did not show any significant change in SP either with concentration or with time. Plain NPs showed a continuous decrease in SP until 10 μg/mL concentration, after which SP remained unchanged. The maximum change in SP for all types of particles occurred within 10 min after particle injection.

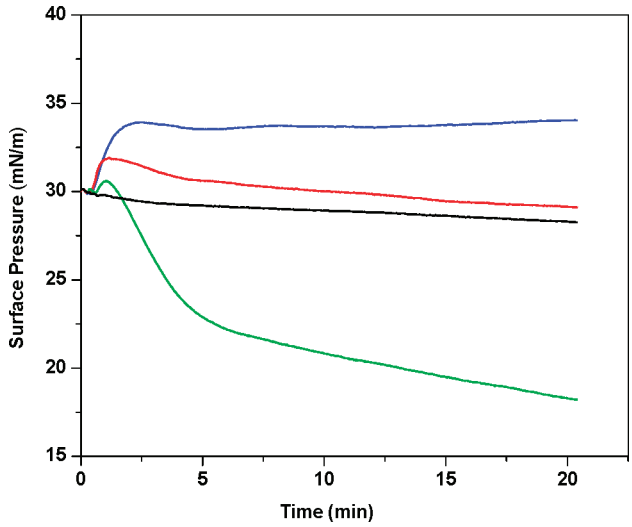

Figure 2. Effects of NP surface groups on surface pressure of endothelial cell model membrane. A 50 μL portion of a diluted suspension of NPs was injected using a Hamilton digital microsyringe below the EMM through the injection port without causing disturbance to the membrane, and change in SP was recorded immediately. NP size = 60 nm. Concentration = 10 μg/mL. Key: blue, aminated; red, carboxylated; green, plain; black, water control without NPs.

Effects of NP Surface Group on the Isotherm of the Endothelial Phospholipid Mixture. This part of the study was carried out to determine the degree of penetration of NPs into the membrane. In the presence of aminated NPs vs plain NPs, the isotherm differed significantly as compared to that of the lipid mixture alone, but the isotherm remained unaltered in the presence of carboxylated NPs (Figure 4). For example, the mmA of the lipid mixture at $\leq$30 mN/m SP was higher in the presence of aminated NPs than in the lipid mixture alone or in the presence of other types of NPs. However, the isotherm pattern changed with further compression and shifted to a lower mmA for plain NPs with respect to the isotherm of the lipid mixture alone. The collapse of the isotherm for the lipid mixture was observed at 55 Å^2 mmA; the same was true in the presence of carboxylated NPs (55 Å^2 mmA).

Effects of NP Surface Group on Interaction with EMM in the Presence of FBS. FBS alone was seen to gradually decrease the SP of the membrane (Figure 5a). Aminated NPs sharply increased the SP within 2 min prior to reaching a pseudoplateau at ~35 mN/m with time, which is almost the similar effect seen with NPs without FBS (Figure 2). However, the presence of FBS completely masked the SP effect of carboxylated and plain NPs (Figure 2 vs Figure 5a). As can be seen the change in SP with these NPs was almost the same as that seen with serum alone.

Effects of NP Size on Interaction with EMM. The general trend was that smaller NPs increased the SP, the magnitude of the increase varying with the surface characteristics of the NPs, whereas larger NPs either did not change SP (carboxylated NPs) or reduced it (plain NPs). An interesting observation was that the increase in SP with 20-nm plain NPs was reversed with larger NPs ($\geq$60 nm; Figure 5b-iii). Furthermore, 20-nm plain (Figure 5b-iii) and 60-nm aminated NPs (Figure 5b-i) retained their effects on SP in the presence of FBS, whereas plain NPs ($\geq$60 nm; Figure 5b-iii) and carboxylated NPs (Figure 5b-ii) showed the same effect as that of FBS. Because of the non-availability of 20-nm aminated NPs, we could not compare the effect of that size of aminated NPs on SP with the same sized plain and carboxylated NPs.

Effects of NP Surface Group on Interaction with DPPC and DPPE Model Membranes. Changes in SP varied with membrane composition and also with surface characteristics of NPs. All of the model membranes showed an increase in SP with aminated NPs, but the increases seen with DPPC and DPPE model membranes were greater and took longer to reach the plateau state than increases seen with EMM (Figure 6a,b). The interaction patterns of plain NPs with DPPC (Figure 6a) and DPPE (Figure 6b) membranes differed, but the pattern observed with DPPC membrane was more similar to that seen with EMM (Figure 2). Carboxylated NPs demonstrated a slight variation in interaction patterns with all three types of membranes, particularly at later time points (Figure 6).

Effects of 20-nm NPs on the Isotherm of the Endothelial Phospholipid Mixture. This part of our study was carried to explain the different behavior of 20-nm-diameter plain NPs compared to the same size carboxylated NPs or larger plain NPs. The isotherm in the presence of 20-nm plain NPs differed significantly and showed higher SPs at different mmA's than that in the presence of the same sized carboxylated NPs, which followed an isotherm pattern almost the same as that of the lipid mixture alone (Figure 7). The isotherm in the presence of 20-nm plain NPs also differed significantly as compared to that seen in the presence of 60-nm plain NPs. These larger NPs showed lower SP with change in mmA as compared to that with 20-nm NPs (Figure 4 vs Figure 7).

AFM Imaging of EMM and Penetration of NPs. The comparative surface morphology of the EMM prior to and after interaction with 20-nm NPs demonstrates the degree of penetration of NPs into the membrane (Figure 8). The phase image of the EMM prior to NP interaction showed condensed domains of lipids surrounded by liquid ordered phase (Figure 8a,b). In the presence of 20-nm plain NPs, the phase image of the membrane showed greater area occupied by the condensed domains than without NPs (Figure 8c). The magnified image (Figure 8d) and the section analysis of the membrane shows ~30 nm spherical structures, which appear to be 20-nm plain NPs embedded in the liquid expanded phase of the EMM (Figure 8e).

Discussion

In the present study, a model for endothelial cell membrane was developed, characterized, and used to study the biophysical interactions with NPs of different surface

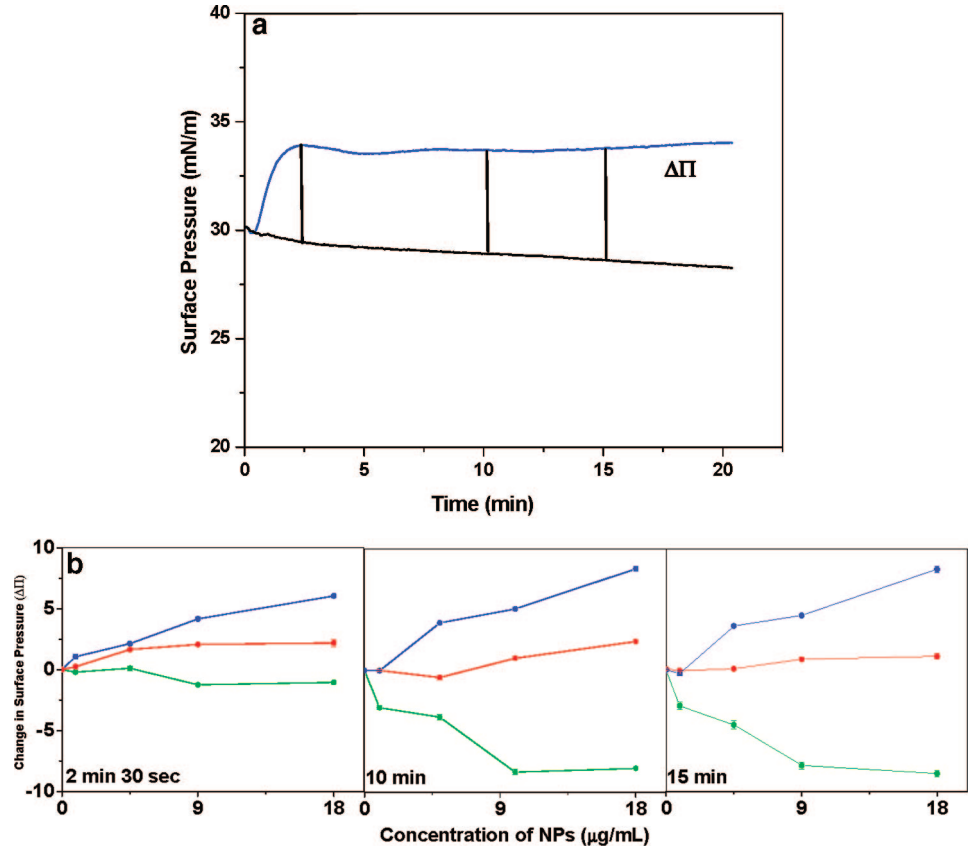

Figure 3. (a) Method used for calculating the change in surface pressure with concentration of NPs of different surface properties. The change in SP ($\Delta\Pi$) with respect to that of the membrane with water injection at particular time points (2 min 30 s, 10 min, and 15 min) was considered, and the difference was plotted against the concentration of NPs. NP size = 60 nm. Concentration = 10 μg/mL. (b) Effects of NP concentration on change in SP of endothelial cell model membrane at different time points following interaction with NPs. NP size = 60 nm. Data as mean ± SEM, $n = 3$. Key: blue, aminated; red, carboxylated; green, plain.

characteristics and sizes. We used the phospholipid composition of the arterial endothelial cell membrane to form the EMM because NPs, following intravenous injection and interaction with serum proteins, would first interact with endothelial lining. Furthermore, endothelium is the target for drug delivery in many vascular disease conditions.[25]

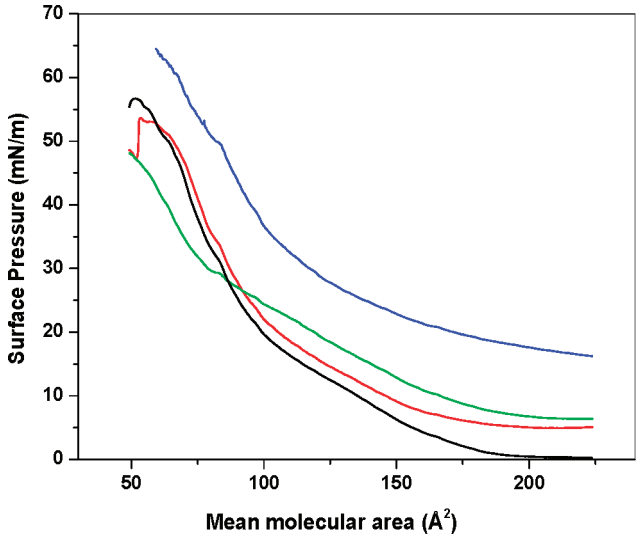

Figure 4. The $\pi-A$ isotherm of the endothelial cell model membrane lipid mixture in the presence of NPs with different surface groups. NP size = 60 nm. Concentration = 10 μg/mL. Key: blue, aminated; red, carboxylated; green, plain; black, water control without NPs.

First, we studied the $\pi-A$ isotherm and compression–expansion of the endothelial lipid mixture to ensure that a stable monolayer is formed at 30 mN/m SP. This SP is reported to be equivalent to the lateral pressure of human erythrocyte cell membranes;[26] hence, the EMM under these conditions is expected to mimic the conditions that exist within biological membranes. The high SP at the point of collapse (~55 mN/m) of the lipid mixture indicates that the formed EMM monolayer is stable and the phospholipids are at the interface until reaching 30 mN/m SP. The overlapping of the expansion isotherm with the compression isotherm demonstrates the respreading ability of the phospholipid monolayer, which indicates that there is no aggregation or

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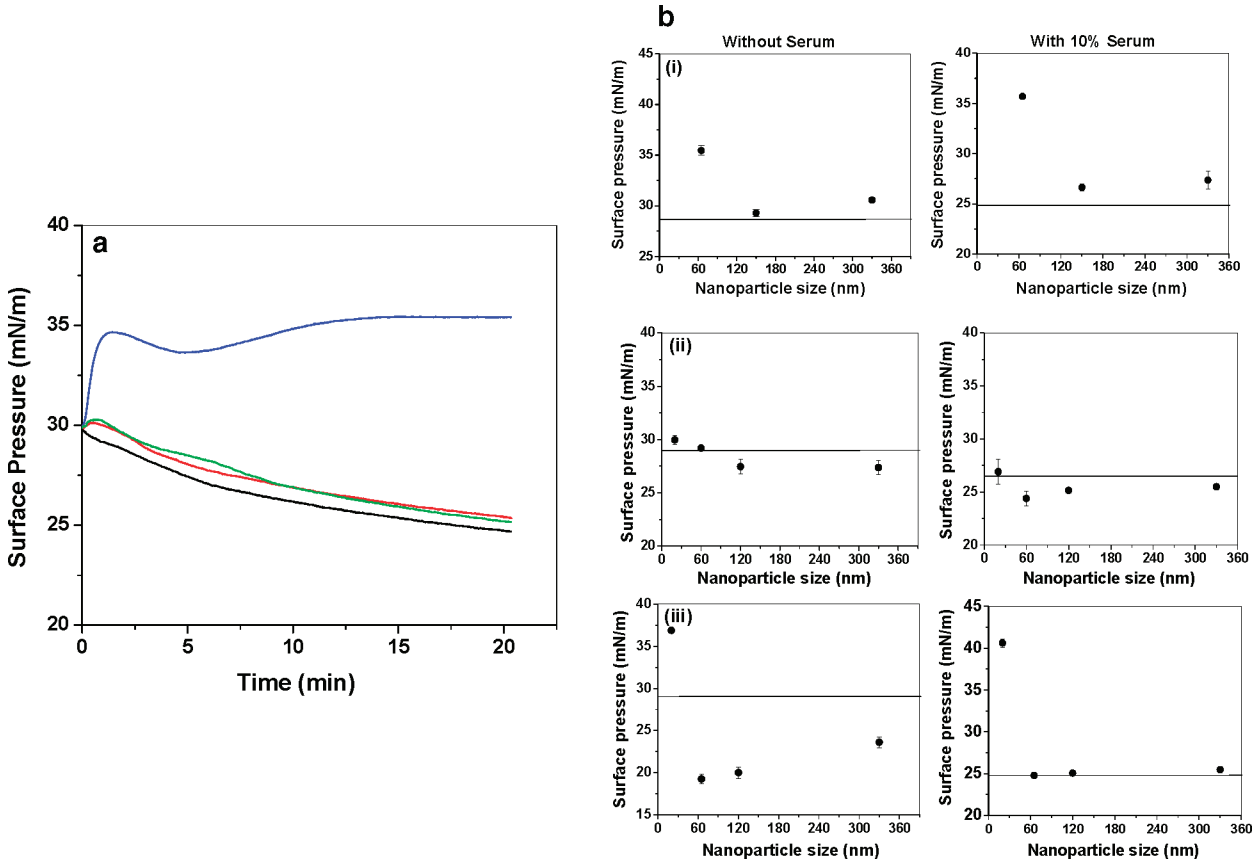

Figure 5. (a) Effects of NP surface group on SP of endothelial cell model membrane in the presence of FBS. Key: blue, aminated; red, carboxylated; green, plain; black, a 10% FBS solution as a control without NPs, NP size = 60 nm. Concentration = 10 μg/mL. (b) Effects of NP size on the SP of endothelial cell model membrane. SP value at 20 min from the SP vs time plot was plotted against NP size with and without FBS. Data for (i) aminated NPs, (ii) carboxylated NPs, (iii) plain NPs. The horizontal line in the graph indicates SP of the endothelial cell model membrane with water or FBS (without NPs) injection as respective controls for interaction studies with NPs in the presence or absence of FBS. NP concentration = 10 μg/mL. Data as mean + SEM, $n = 3$.

entanglement of lipid chains at the interface. The stable nature of the EMM is important to ensure that changes in SP of the membrane are indeed due to interaction with NPs.

In the Langmuir monolayer, SP depends on mmA and indicates the arrangement and the state of phospholipids at the interface. For instance, a low SP ($\sim$0 mN/m) at high mmA ($\sim$150 Å^2 mmA) indicates that phospholipid molecules are far apart from each other at the interface, i.e., in the gaseous state. High SP (50 mN/m at low 55 Å^2 mmA) indicates that phospholipids are compressed and exist in a solid state. Since SP values depend on mmA, changes in SP at a constant mmA with time would provide information about the interaction of NPs with EMM.

In general, the phospholipid condensation at the interface could occur either because of the amount of penetration of molecules, as that would reduce the available area for lipids, or electrostatic interactions which would reduce the repulsive forces between the lipid molecules, allowing them to come close to each other to form a condensed membrane. Penetration of molecules into model membranes has been shown for small hydrophobic drug molecules,[21,27,28] partial hydrophobic molecules such as cell penetrating peptides,[29,30] and surfactant.[22] Electrostatic interactions with phospholipids have been shown for charged hydrophilic molecules such as DNA[31] and dextran sulfate.[32] The loss of phospholipids into the subphase has been observed with studies involving

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(31) Gromelski, S.; Brezesinski, G. DNA condensation and interaction with zwitterionic phospholipids mediated by divalent cations. *Langmuir* **2006**, *22*, 6293–6301.

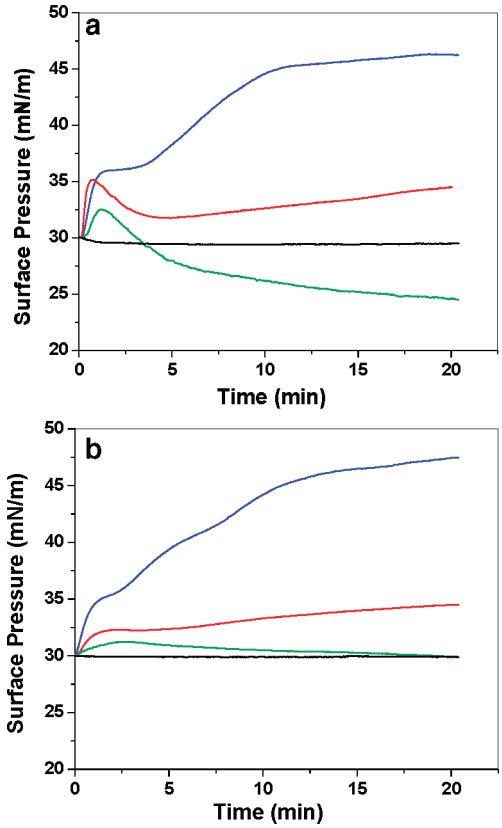

Figure 6. Change in SP of (a) DPPC model membrane and (b) DPPE model membrane following interaction with NPs. NP size = 60 nm. Concentration = 10 μg/mL. Key: blue, aminated; red, carboxylated; green, plain; black, water control without NPs.

interaction of long-chain biopolymers such as chitosan.[33,34] No change or minimal change (either decrease or increase) in SP values from 30 mN/m demonstrates the lack of interaction of molecules with the membrane.[35–37]

Based on the findings noted above, the comparative changes in SP of the membrane observed in our studies can be

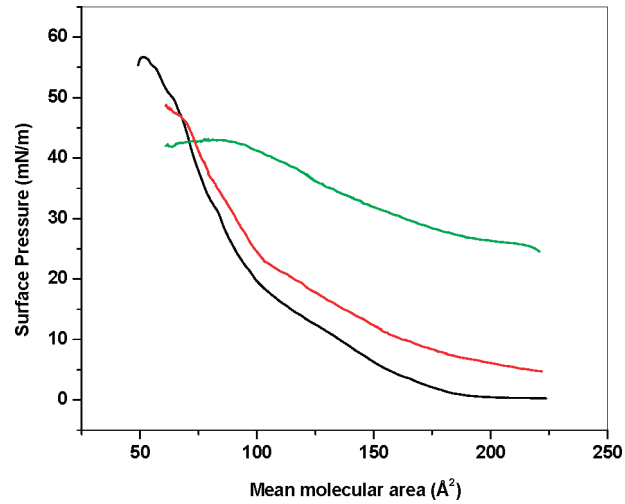

Figure 7. $\pi-A$ isotherm of the endothelial cell model membrane lipid mixture alone (black line) and in the presence of 20-nm plain NPs and 20-nm carboxylated NPs (green and red lines, respectively). NP concentration = 10 μg/mL.

interpreted as follows. The increase in SP of the membrane in the presence of aminated NPs indicates the condensation of phospholipids at the interface, whereas the decrease in SP in the presence of plain NPs indicates the loss of phospholipids from the interface into the bulk (Figure 2). In the case of aminated NPs, the increase in SP could be because of the electrostatic interactions with the negatively charged (PO_4^-) head groups of phospholipids, causing condensation of the membrane. In the case of plain NPs, the decrease in SP could be caused by the interactions of hydrophobic chains of phospholipids with hydrophobic plain NPs, which then mobilize the phospholipid molecules from the interface into the subphase. No change in SP in the presence of carboxylated NPs indicates a lack of interactions with phospholipids in the membrane (Figure 2). The schematic representing interactions of NPs of different surface properties with EMM is shown in Figure 9. Since there was no interaction with carboxylated NPs, these NPs did show a significant change in SP with concentration, whereas the aminated and plain NPs demonstrated concentration-dependent changes in SP (Figure 3b). The effects of differing concentrations of NPs on SP was greater during the initial time points, which then reached almost a steady state, suggesting a saturation effect of interaction of NPs with the membrane.

Since the changes in SP may not provide information on whether the increase in SP is due to electrostatic interaction only or is also due to the penetration of NPs, we studied the isotherms of the lipid mixtures of EMM in the presence and absence of NPs (Figure 4). Other investigators have determined the penetration ability of pluronic into DPPC and DPPE model membranes via similar experiments. In these studies, it has been suggested that pluronic penetrates DPPC membrane at a high mmA or low lipid density (this arrangement of phospholipids represents damaged cell membranes), whereas pluronic squeezes out of the membrane at low mmA or high lipid densities (~30 mN/m SP).[35–37]

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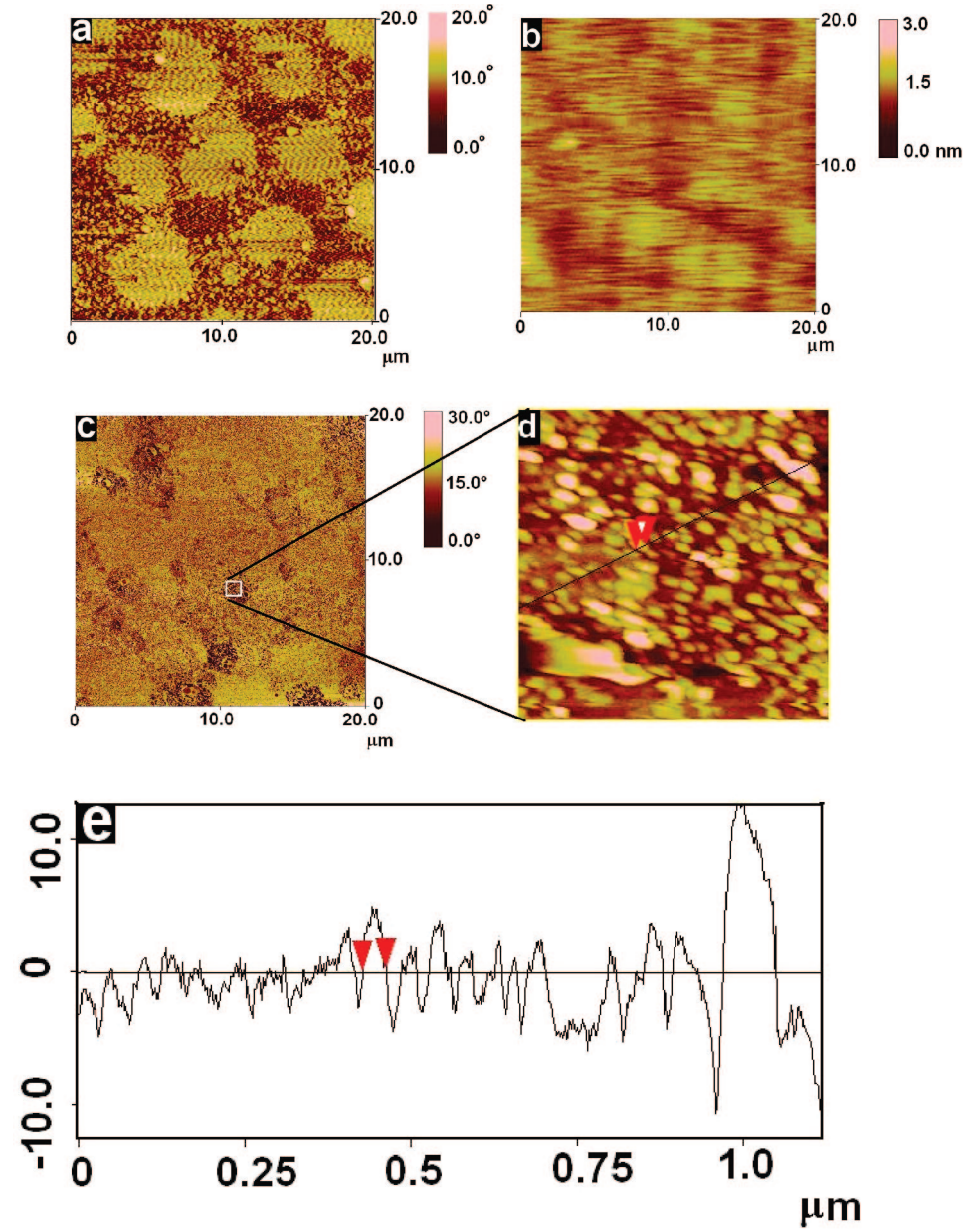

Figure 8. AFM phase (a) and height (b) image of endothelial cell model membrane transferred onto glass substrate. (c) Endothelial cell model membrane transferred following interaction with 20-nm plain NPs. (d) Height image of the magnified part of the image following interaction with NPs. (e) Section analysis of image (c) across the black line. The width of the spherical structures is ~30 nm.

The results of our isotherm experiment demonstrate that all three types of NPs we used can penetrate the lipid mixture monolayer at lower lipid densities (below 10 mN/m SP); however, at higher lipid densities (30 mN/m SP), only aminated NPs seem to remain in the monolayer. This conclusion is evident from the shift in the isotherm toward higher mmA (Figure 4). Carboxylated NPs seem to squeeze out of the monolayer; hence the isotherm in their presence is almost similar to that without NPs. In the case of plain NPs, the SP of the lipid mixture was lower at 85 $Å^2$ mmA than that for the lipid mixture without NPs, indicating the loss of phospholipid molecules from the interface into the subphase.

It seems that FBS itself destabilizes the membrane, as its presence showed a slow decrease in SP with time (Figure 5a). FBS contains various proteins with both positively and negatively charged surface groups that can interact with the charged head groups of phospholipids in EMM and thus can lead to destabilization of the membrane.[38,39] In general, we observed that the presence of FBS does not influence the effect of smaller plain NPs and aminated NPs on SP but

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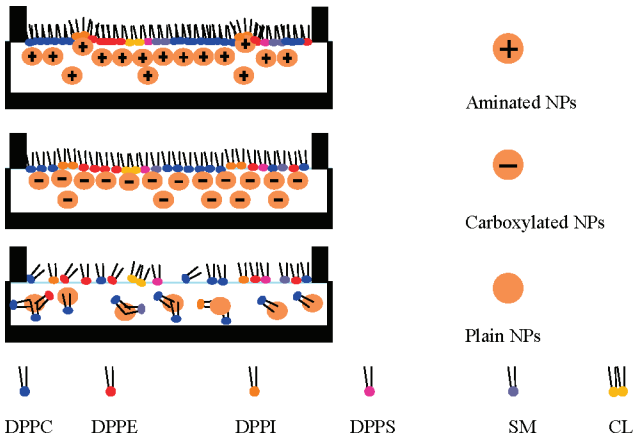

Figure 9. Schematic representation of the interaction of NPs of different surface groups with endothelial cell model membrane. Aminated ($R\text{-}NH_2$) NPs interact with the negative (PO_4^-) charge of the polar headgroup of phospholipids, resulting in condensation of phospholipids at the interface, thus increasing SP. Carboxylated (-COOH) NPs do not show a strong interaction with phospholipids; therefore, SP of the membrane remains unchanged. Plain NPs are hydrophobic and interact with hydrophobic chains of phospholipids, resulting in the displacement of phospholipids from the interface to the subphase which causes a decrease in SP and destabilization of the membrane.

does influence that of larger plain NPs (Figure 5b). The difference in the effects of serum with size and surface groups could be due to the difference in the adsorption pattern of proteins on the surface of the NPs. It has been reported that the type and amount of protein adsorption differ with surface group, surface charge density,[40,41] and size of NPs.[42] Our results suggest that serum interaction masks the surface properties of large NPs but not that of small NPs. It has been shown that lysozyme adsorbed onto smaller NPs retains its native structure and function in comparison to its adsorption onto larger NPs,[42] suggesting the difference in adsorption pattern of protein with size of NPs. Based on our results, we speculate that protein links linearly to small NPs without affecting the interaction properties of NPs with the membrane, whereas protein aggregates on large NPs, masking their surface properties and thus influencing the interaction with the membrane. This difference in adsorption pattern of protein is suggested to be due to the greater surface curvature of small NPs. The effect of size or concentration on change in SP does not seem to be the effect of change in total surface area of NPs that comes in contact with membrane since there is neither a steady increase in SP with an increase in concentration of NPs (Figure 3), nor a decrease in SP with an increase in particle size (Figure 5). We also rule out the possibility of the stabilizing agent present (1% sodium dodecyl sulfate, SDS) in the original stock suspension of plain and carboxylated NPs affecting the SP of membrane. If it would have been the effect of surfactant, NPs would have shown the same effect on SP of membrane irrespective of their surface chemistry but their effect is significantly different (Figure 2). Further, we determined the SP effect of plain (120 nm) NPs which are available in surfactant-free medium, and it was the same as that of the NPs diluted from the stock available in 1% SDS. The surfactant in NP suspension perhaps did not influence SP because of its significant dilution (10,000 times) at the NP concentration present in the trough. Unfortunately, NPs of all size and surface chemistry are not available in surfactant-free medium.

We have also measured the change in SP of buffer without the lipid monolayer for different NPs at 10 μg/mL concentration. Irrespective of their surface characteristics, NPs showed a rapid increase in SP, the magnitude of which was greater for aminated NPs (increased from 0 to 5 mN/m) than that for the carboxylated or plain NPs (increased from 0 to 1–2 mN/m). The rapid increase in SP returned to 0 mN/m within 10 min after NP injection. This pattern of change in SP seen with buffer only was distinctly different than that seen with NPs in the presence of EMM, clearly suggesting that NP−membrane interactions are independent of the intrinsic surface active behavior of NPs.

The study of interaction of NPs with DPPC and DPPE model membranes allowed us to determine which lipids in the EMM may be involved in interaction with NPs. The results show that the membranes formed with single phospholipids interact differently than with EMM, which is formed with a mixture of phospholipids. DPPC and DPPE model membranes showed a greater degree of condensation than EMM, which is evident from the greater change in SP in the presence of aminated NPs than EMM. The low degree of condensation of EMM could be due to the presence of other minor lipids (PI, SM, CL), which are unsaturated hydrocarbons and are known to hinder the condensation of phospholipids.[43] The difference in change in SP was also seen with carboxylated NPs. The slow increase in SP could be due to the electrostatic interaction between carboxylated surface groups on NP surface with positively charged head groups of DPPC and DPPE lipids. In contrast, the slow decrease in SP with EMM could have been due to the repulsive force between the carboxylated groups and anionic hydrophilic headgroups of DPPS phospholipid present in

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EMM. The change in SP of plain NPs interacting with DPPC and EMM membranes (i.e., destabilization of the membrane) was similar, whereas no significant change with DPPE membrane was seen. This difference in level of interaction could have been due to the difference in the arrangement of phospholipid head groups in the membrane. In the case of DPPC, the polar head groups are arranged at a tilted angle, whereas the polar head groups of DPPE are arranged vertically at the surface.[44] Therefore, the hydrophobic chains of DPPC are available for interaction with hydrophobic plain NPs; the hydrophobic chains of DPPE are not available for interaction because of their tilted arrangement at the interface. The results thus show that membrane composition influences interactions with NPs.

Preetha et al.[45,46] observed a similar discrepancy in the interaction of paclitaxel with monolayer model membranes formed with lipids extracted from cancer tissues/cells and normal cell lipids and the membrane formed with DPPC. Zhirnov et al.[18] also reported that interaction of pluronics with model membranes depends on the membranes' lipid composition. Hence, it is important that a membrane with the appropriate lipid composition be used to mimic the conditions to which nanomaterials will be exposed following their *in vivo* administration.

Although the isotherm experiment speculates penetration of NPs into the lipid mixture at low lipid density, it is less likely to happen when the interactions are studied with compressed membrane because the particle size is too large (60 nm) to penetrate through the closely packed phospholipid membrane. Therefore, the most likely mechanism for increase in SP with aminated NPs could be the electrostatic interactions of cationic surface R-NH_2 of NPs with the negatively charged phosphate head groups of lipids. The model proposed in Figure 9 also explains the greater increase in SP with smaller aminated NPs (Figure 5). Since surface-to-volume ratio increases with decreasing size, the number of R-NH_2 groups available for interaction with the membrane increases. A similar effect was observed on cells with polycationic polymers, in which the difference in surface charge density is due to the difference in the molecular structure.[47] We speculated that the small plain NPs penetrate the membrane, whereas the larger NPs instead bind to lipids at the interface and displace them into the subphase, thus destabilizing the membrane. To support the above mechanism, we studied the compression isotherm as described above in the presence of 20-nm plain NPs and compared that with the isotherm obtained with 60-nm plain NPs (Figure 7 vs Figure 4). The results demonstrate that 20-nm NPs remain in the membrane even at high SP levels, as is evident from the higher SP of the membrane in their presence. This effect does not appear to be the effect of size because carboxylated NPs of the same size did not show the same high SP (Figure 7).

AFM analysis further confirmed the penetration of 20-nm plain NPs into the EMM. The phase image of the EMM alone showed bright island-like structures surrounded by dark regions. Based on the literature,[31,39] these bright structures are assigned to the condensed phase and the dark area to the liquid-expanded phase of phospholipids. The phase image of the EMM shows island-like structures which are separated clearly from each other (Figure 8a), whereas these structures following interaction with NPs become more indistinguishable from each other, suggesting further condensation of the membrane in the presence of NPs (Figure 8b). The magnified image of the liquid-expanded phase shows spherical structures (Figure 8c), and section analysis revealed the presence of NPs in the membrane (Figure 8d). These results support the assumption that 20-nm plain NPs cause condensation of the membrane because of their penetration into the liquid-expanded phase of the membrane. Plain 60-nm NPs showed a disrupted monolayer following the interaction study, therein supporting our notion that these particles bind to the lipids at the interface and displace them into the subphase, thus destabilizing the membrane. Other techniques, such as *in situ* Brewster angle microscopy or X-ray reflectivity, where membrane transfer is not required, could provide more insight into the interactions of NPs with the membrane.

The NP-EMM interactions observed in this study show a significant analogy to what is known for nanomaterial characteristics and their interactions with cells and *in vivo* disposition properties. For example, it is known that small cationic and plain NPs have greater cellular uptake than anionic and large NPs.[13] Our study also demonstrates a greater increase in SP with small NPs. It is also known that small NPs stay for a longer time in the circulation than larger NPs, primarily because of the difference in protein adsorption. It is suggested that the protein adsorbed onto small NPs remains in its natural form and hence they are not recognized by the RES.[14] Based on the SP effect, our studies also suggest that interaction of protein with smaller NPs is different than that with larger NPs, and does not affect their interaction properties with the membrane.

Despite these analogies, it must be recognized that the interaction of NPs with the membrane is the first step in the complex process of cellular uptake and intracellular trafficking of nanomaterials. Further, our model membrane may not represent all the aspects of live cell membrane. For example, it is a monolayer of lipids rather than a bilayer and lacks anchoring membrane proteins present in live cell membrane. In addition, the model used does not take into account the hydrophobic interaction between the lipid tails and proteins. Hence, the challenge will be to develop a model membrane that would mimic the many aspects of a living cell membrane. It is therefore important to develop an

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accurate correlation between the data obtained from the membrane interaction and interactions with cells/tissue to interpret the data from model membrane.

Nanocarrier systems are usually characterized for size and ζ potential, which may not predict their interaction behavior with biological membranes. For example, 20 nm carboxylated and plain NPs have almost similar ζ potential ($\zeta = -40.46$ vs -40.12 mV), but the $\pi-A$ isotherm of the lipid mixture in their presence significantly differs (Figure 7). We believe that the biophysical interactions of NPs with appropriate model biomembranes could provide more pertinent information that can possibly predict NP interactions with cells/tissue and their disposition characteristics *in vivo*; and could also be used in optimizing the characteristics of nanomaterials for specific biological applications.

Conclusions

Based on the change in SP, the interaction of NPs with the EMM significantly depends on their surface characteristics and sizes. In general, small aminated NPs and plain NPs have greater interactions with the EMM than do carboxylated and large, plain NPs. Further, FBS does not seem to influence the interaction of small NPs with the membrane. Our overall results suggest that interaction with model membranes could be a useful parameter by which to characterize nanomaterials and this factor can be explored for developing effective nanomaterials for biomedical applications.

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