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Interactions of Poly(amidoamine) Dendrimers with Surfactant Lung Surfactant: The Importance of Lipid Domains

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Abstract

The interaction of generation 5 (G5) and 7 (G7) poly(amidoamine) (PAMAM) dendrimers with mica-supported Surfactant bilayers is studied with atomic force microscopy (AFM). In these experiments, Surfactant forms distinct gel and fluid domains with differing lipid composition. Nanoscale defects are induced by the PAMAM dendrimers. The positively charged dendrimers remove lipid from the fluid domains at a significantly greater rate than for the gel domains. Dendrimer accumulation on lipid edges and terraces preceding lipid removal has been directly imaged. Immediately following lipid removal, the mica surface is clean, indicating that lipid defects are not induced by dendrimers binding to the mica substrate and displacing the lipid.

Introduction

Understanding the interaction between nanoparticles and the lung lining is an ever-increasing concern as the amount of airborne particulate matter introduced into the environment by human activity grows.¹ Nanoparticles have been implicated in a number of acute and chronic medical conditions, particularly those associated with inflammation and cardiovascular disease.^{2–4} A better understanding of the mechanisms by which nanoparticles penetrate the lung lining and enter the lungs would allow for better treatment and prevention of diseases.

Surfactant is an excellent model system for investigating the mechanisms of nanoparticle transport and toxicity in the lungs because it mimics the composition and function of the

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Supporting Information **Available**: Additional data including crops of regions of interest and 3D renderings of figures throughout the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

alveolar lining while being a relatively well-defined experimental system. Additionally, Survanta is a widely used clinical surfactant for the treatment of Respiratory Distress Syndrome (RDS) making its choice particularly relevant.^{5,6} Survanta's ability to form stable monolayers at the alveoli-air interface that spread with alveoli expansion and contraction to maintain a low surface tension are critical for proper lung function, which is compromised in RDS due to an incomplete surfactant layer. Both mechanistic and in vivo studies have been performed addressing these issues, making Survanta one of the best-characterized lung surfactants available.^{7–9}

The two major lipid components in Survanta, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG), have melting temperatures significantly above and below room temperature, 41 and -2°C respectively.¹⁰ When deposited onto mica, this difference in transition temperature leads to distinct fluid and gel domains that exhibit little structural or phase changes over time at room temperature. These distinct domains allow for direct comparison between nanoparticle interaction with fluid and gel phases. Second, the presence of fatty acids (palmitic) and proteins, observable with atomic force microscopy (AFM),^{11,12} brings us closer to the level of complexity seen in actual cell membranes.

Nanoscale disruption of lipid membranes by charged polymer-based nanoparticles is well documented in the literature.^{13–19} These studies demonstrate that charged nanoparticles disrupt biological membranes; however, they leave many open questions regarding the mechanism of disruption, including the role of headgroup charge,^{13,16} the influence of lipid phase,^{13,15} and the effects of cholesterol, proteins and other membrane components. In 2005, Mecke et al. addressed the question of phase by observing that only lipid in the fluid phase was removed by amine terminated poly(amidoamine) (PAMAM) dendrimers.¹⁵ A number of mechanisms for membrane disruption have been suggested. In one such model, the lipids encapsulate the polymer in a lipid vesicle.¹⁶ More recently, Gewirth and colleagues suggest the possibility that disruption of the electrostatic interactions between the charged mica substrate and the lipid bilayer lead to lipid removal.^{18,19}

PAMAM dendrimers were chosen as the model nanoparticles for interaction with Survanta because of the well-controlled size, excellent polydispersity (1.01), and well-defined surface chemistry.^{20–22} Under AFM imaging conditions, unbuffered and neutral pH, all primary amine groups of the PAMAM dendrimers are expected to be protonated.^{23,24} Space filling models of G5 and G7 dendrimers are shown in Figure 1.

There are three key results reported in this paper. First, the fluid domain is removed more than an order of magnitude faster than the gel domain. Second, dendrimer accumulation on lipid edges and terraces preceding lipid removal has been directly imaged for both fluid and gel domains and all tested dendrimer generations for the first time. Third, immediately following lipid removal the mica surface is clean. This indicates that lipid defects are not induced by dendrimers binding to mica and displacing the lipid.

Materials and Methods

Survanta was generously provided by Ross Laboratories (Columbus, OH) and stored at 4°C . Survanta, consists of 25 mg/mL of phospholipids: 44–62% 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 13–45% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[Phospho-*rac*-(1-glycerol)] (POPG), 2–7% triglycerides, 5–14% fatty acids most of which is Palmitic acid, and less than 4% surfactant proteins B and C all suspended in a 0.9 M NaCl solution. For these experiments, Survanta was diluted by a factor of 10 with PBS buffer (Sigma-Aldrich, St. Louis MO) to a phospholipid concentration of 2.5 mg/mL. 80 μL of the dilute Survanta

was warmed to 37 °C and deposited onto freshly cleaved mica surfaces roughly 1 cm² in size forming bilayers via the vesicle fusion method. Surfaces were allowed to incubate for at least a half-hour in a humid environment at 37 °C, which is above the observed transition temperature for both domains, ensuring layers formed with minimal defects. This observed transition temperature is below that of pure DPPC because both the fatty acids and POPG present in the DPPC enriched domain lower and broaden the observed transition temperature.

Samples were washed with ultra pure water (NERL Diagnostics, East Providence, RI) to remove any excess lipid and transferred to either a Nanoscope IIIa Multimode scanning probe microscope from Digital Instruments equipped with an “E” scanner (Veeco Metrology Group, Santa Barbara, CA) or a PicoPlus 5500 AFM equipped with a multipurpose small scanner from Molecular Imaging (Agilent, Chandler, AZ). Bilayers were kept in ultra pure water during imaging. Images were taken in tapping mode using silicon nitride cantilevers (DI model NPS, spring constant 0.32 N/m, length 100 μ m) with a (3 μ m)² scan size, 512 scans per frame, between 2–4 Hz (typically 3 Hz). All imaging was done at room temperature, nominally 25 °C, which led to one domain, mostly DPPC, in the gel phase and the other, mostly POPG, in the fluid phase.

Generation 5 and 7 PAMAM dendrimer were purchased from Dendritech Inc. To remove lower molecular weight impurities and trailing generations, the dendrimer was dialyzed with a 10,000 molecular weight cutoff (MWCO) membrane against deionized (DI) water for two days, exchanging washes every 5 h. The purified dendrimer was lyophilized for three days resulting in a white solid. The number average molecular weight (25 280 g/mol) and PDI (1.015 $\pm$ 0.010) was determined by GPC. Potentiometric titration was conducted to determine the average number of primary amines (108) as previously described.²⁴ Generation 7 PAMAM dendrimer was purchased from Dendritech Inc. To remove lower molecular weight impurities and trailing generations the dendrimer was dialyzed with a 50 000 MWCO membrane against DI water for two days, exchanging four washes. The purified dendrimer was lyophilized for three days resulting in a white solid. The number average molecular weight (105 600 g/mol) and PDI (1.053 $\pm$ 0.012) was determined by GPC. Dendrimer solutions, made with ultra pure water, were injected into the fluid cells of either instrument while imaging until the concentration within the cell was between 80 and 150 nM. In either instrument, the injected volume was much less than the volume of the cell, preventing significant changes to the resonance frequency of the tip.

Results and Discussion

Observed Domain Structures of Surfactant Supported Lipid Bilayers

A typical AFM image of a Surfactant bilayer on mica is illustrated in Figure 2. Domain A, shown on the left and right edges in light gray is predominantly composed of zwitterionic DPPC in the gel phase.²⁵ Domain B, the darker gray in the middle of Figure 2 is predominantly the anionic POPG in the fluid phase.¹² These assignments were made by comparing observed heights to published AFM values for DPPC³⁰ and a POPG/POPE¹⁵ mixture. The chemical structures of both lipids are shown in Figure 3. A defect present in the middle of Domain B reveals the underlying mica substrate shown in black. Finally, the speckled patterns seen in the interior of Domain A are due to the presence of palmitic acid and surfactant proteins as noted by Ding et al.^{7,26} These speckled features are only seen in the interior of large Domains A, while excluded from the edges of Domains A and entirely from Domains B. The observed thickness of Domain A is 5.2 nm, Domain B is 3.6 nm, and the speckled features are 5.6 nm in height.

Role of Fatty Acids and Proteins

An initial question addressed by these experiments was whether or not fatty acids and proteins present in Survanta could cause surface features that act as nucleation sites for lipid disruption. As shown in Figure 2, these features were observed in the Survanta bilayer as speckled regions; however, no activity in or near these speckled regions was detected. This suggests that at these concentrations, interactions between the dendrimer and the fatty acids and proteins do not lead to disruption of the membrane.

Faster AFM Scanning

A key improvement in this paper is a significant increase in the imaging speed of the AFM during experiments. Prior efforts by our group have produced images with $(1\ \mu\text{m})^2$ scan sizes, 256 lines per scan, at 1 line per second.^{13–17} In the current work, the limits of these commercial AFM systems have been pushed to increase the tip velocity significantly, allowing for $(3\ \mu\text{m})^2$ scan sizes, 512 lines per scan, at 3 lines per second. With these imaging conditions, 66% of the lateral resolution of previous works is maintained; however, as the pixel size, 5.9 nm, is significantly less than the radius of curvature of the probes, ~ 20 nm, the same fundamental resolution limit as before exists. For a spherical tip, the smallest feature height or depression that could be resolved by a single pixel is $2(R_c^2 - (0.5 \times \text{pixel width})^2)^{1/2}$. For a 20 nm tip with a pixel size of 5.9 nm (these experiments), the feature height resolvable is 0.2 nm. For a 20 nm tip with a pixel size of 3.9 nm (previous experiments), the feature height resolvable is 0.1 nm. As this difference is within the experimental noise of the system, no resolution is lost. The improvement comes from the fact that 9 times the total area is scanned 1.5 times as fast as what was previously done. This greatly enhances the number of events that can be followed within a given field of view, significantly increasing the probability that an intermediate state, such as dendrimer accumulating on lipid, can be observed.

Domain Dependence of PAMAM Dendrimer Interaction

For the Survanta bilayer, removal of lipid by the positively charged PAMAM dendrimers was observed for both Domain A and Domain B; however, Domain B, primarily consisting of a fluid-phase POPG lipid bilayer with a negatively charged phosphatidyl glycerol headgroup, was preferentially disrupted and removed by both G5 and G7 PAMAM dendrimer.

In Figure 4, almost exclusive removal of Domain B by G5 PAMAM dendrimer is observed. Most of the lipid is removed from the edges of existing defects, shown with blue arrows; however, there are two instances of lipid removal (Figure 4D), shown with purple arrows, which are not extensions of existing observable defects. In other images (Supplemental Figure 1), removal of Domain A by G5 PAMAM dendrimer is more apparent. Figure 5 shows that Domain B is preferentially removed by G7 PAMAM dendrimer. Comparing the amount of lipid removed in a fixed amount of time from Domain A, indicated by blue arrows in Figure 4D to Figure 4E, to the amount removed from Domain B shows at least an order of magnitude increase. Experimental limitations prevent examination of the steady-state equilibrium of the system, thus precluding conclusions regarding the overall thermodynamics of the interaction. However, it is clear that the energy barrier to remove lipid, which sets the kinetic rate, is much higher for Domain A than Domain B. This could be due to a shielding effect from the positively charged choline termination reducing interaction with the phosphate group, the increased ordering of the gel phase DPPC or, most likely, a combination of the two.

We believe that a combination of electrostatic attraction and hydrophobic effects drive the disruption of the membrane. In previous reports, exclusive removal of fluid domains, similar

to Domain B, was observed.^{13,15} In this experiment, significant removal of gel phase material has been observed because of the presence of two large, distinct domains which have transition temperatures significantly above and below experimental temperatures. In prior reports, imaging was done near the transition temperature of DMPC which led to a smaller fraction of material in the gel phase, which reduces the probability of observing an interaction with the gel domain.^{27,28}

Disruption Models

Lipid removal by charged polymers generally falls into one of two categories, adherence and disruption. In adherence mechanisms the charged polymer, in this case PAMAM dendrimers, attaches to the lipid head groups through electrostatic interactions and the hydrophobic effect maximizes exposure between the hydrophobic tails of the bilayer and the hydrophobic core of the dendrimer. These forces disorder the membrane and allow for lipid removal. Disruptive methods will either disrupt the head groups, tails, or, as suggested by Gewirth and colleagues, the electrostatic interactions between the bilayer and mica.^{18,19} Regardless of the mechanism of disruption, these methods do not always result in encapsulation of the polymer. In Figure 6, lipid removal from the fluid phase by G7 PAMAM dendrimer (nominally 512 surface amines) is shown in two $(3\ \mu\text{m})^2$ scans ~ 2 min apart. Looking at the lipid removed between images Figure 6C and Figure 6D at the upper edge of the defect, highlighted with a teal arrow, shows that the lipid is removed cleanly. That is, only the smooth mica that underlies the bilayer is observed. Previous studies have shown G5 dendrimer adhering to mica substrate $\sim 1\text{--}2$ nm tall and $10\text{--}15$ nm in diameter.²⁹ If lipid were removed because the dendrimer bound to the mica and displaced the lipid, the dendrimer should remain attached to the mica. At the size scale in Figure 6, if the dendrimer was still attached to the mica a rough texture would be observed. The lack of this textured surface leads to the conclusion that lipid removal is not due to displacement by dendrimer. As a control, G5 dendrimer, was spin coated onto freshly cleaved mica. The sample was then imaged via tapping mode in water at the same $(3\ \mu\text{m})^2$ scan size and rate as before. Features between 2 and 3 nm tall and 15 nm in diameter are present. This agrees with previous reports. G5 was used as a control for this experiment specifically because the smaller size, relative to G7, would make it more difficult to observe. Because of this, G7 should have been observable in Figure 6D. This provides confidence that the lipid removal is not due to the dendrimer interacting strongly with the mica substrate and displacing the lipid. Additionally, regions of lipid removal by G7 dendrimer from Figures 5 and 6 have been rendered in 3D in Supplemental Figures 2 and 3 to further elucidate this point.

In previous studies, G3 and G5 PAMAM dendrimers have been observed accumulating near the edges of preexisting defects in supported DMPC bilayers, but not with G7.¹⁶ In Figures 4 and 5, dendrimer accumulation preceding hole expansion is highlighted with teal arrows and red boxes. Since very little material accumulates prior to removal, faster imaging speeds and larger image acquisition allows us to capture the intermediate state—where material has accumulated but not yet been removed—more often. Figure 7 shows the phase deflection image of Figure 5D and a line scan through Domain A, Domain B, and material that has accumulated on top of both domains. Phase imaging can be used to distinguish between different materials because “stickier” and “softer” (less elastic) materials will cause a larger phase lag between the driving signal and the deflection signal. In this color scheme, brighter values indicate a stickier or softer material. In this image, regions of dendrimer accumulation, originally identified by topography, are, in fact, regions of different exposed material. Moreover, when compared to the results of molecular dynamics simulations of G3 PAMAM dendrimer interacting with a bilayer made from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) additional evidence for why the dendrimer should be interacting with the upper leaflet of the bilayer is presented. The simulation images, (right side of

Figure 7) show how both the dendrimer and the bilayer deform to maximize their interaction. The increased disorder of the fluid layer explains why the observed phase lag between Domain B and the G7 PAMAM dendrimer is smaller than for the dendrimer and Domain A.³⁰

In order to understand what is going on in these regions where the dendrimers accumulate, boxed regions in Figures 4D and 5D have been selected for closer examination. 3D renderings of these regions are provided in Supplemental Figures 4–6. These regions were selected because they show accumulation of material that precedes lipid removal and these regions are elevated above the underlying lipid with a distinct edge. Multiple line-scans have been taken across these edges in the boxed regions in Figures 4D and 5D so that the line scans start the same distance from the edge. The line scans were averaged to generate representative profiles. This allows for examination of the “step height” going across this edge from one lipid domain to the accumulated material on top of it, in terms of both generation and underlying domain. The step height is plotted in Figure 8 with the height of the reference domain set to zero. What is interesting about this plot is the difference in slope between Domain A and Domain B is independent of dendrimer generation. Factoring in the higher step heights—summarized in Table 1—for Domain A and Domain B, the dendrimer, then, should be able to spread out more and disturb more of the lipid bilayer possibly explaining why lipid is removed faster from the Domain B than Domain A. These conclusions match the molecular dynamics results shown in Figure 7 where the dendrimer on fluid DMPC is more extended and deformed than on the gel layer.³⁰

Considering the information from the phase images, the inaccessibility of the hydrophobic tails to the dendrimer, molecular dynamics simulations showing the dendrimer is on top of the bilayer, our evidence indicating that the dendrimers do not accumulate between the bilayer and mica substrate, and the morphology of the accumulated regions, the conclusion is that the dendrimers are sitting on top of the lipid bilayer or partially intercalated into the hydrophilic head region.

Conclusions

Clinical lung surfactants, Surfacta being one example, provide opportunities to study aspects not traditionally captured in single component lipid bilayer experiments. Questions regarding lipid phase, composition, roles of fatty acids and proteins can all be examined in a relatively controlled environment. In the case of polycationic nanoparticle induced disruptions, lipid-polycation interactions still appear dominant. The data presented supports lipid removal caused by dendrimers adsorbing onto the upper leaflet and interacting strongly with the hydrophilic head groups, disrupting the lipid. For the first time, the adsorption of material has been observed preceding lipid removal in all cases, the rate of which is governed by the underlying domain phase.

An oft asked question is how can nanoscale results be related to global behavior? In this case, how would the domain segregation and preferential disruption of Domain B affect the behavior of the surfactant layer in a living animal? To answer this question, the authors direct the reader to an excellent book, *Lung Surfactant Function and Disorder*, edited by Kaushik Nag.³¹ This book features a very thorough review of the composition, function and structure of surfactants from the nanoscale through the macroscale. The book relates how physical properties at all scales support proper lung function, and how disruptions can lead to diseased states. The reader will find Chapters 6, 10, and 12 particularly relevant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Space filling models of equilibrated generation five (G5) and generation seven (G7) poly(amidoamine) PAMAM dendrimers. The G5 dendrimer has 128 surface amines and an approximate diameter of 5 nm. The G7 dendrimer has 512 surface amines and an approximate diameter of 7 nm.

Figure 2.

This is an example of a well-formed Surfvanta bilayer. The image is $(3\ \mu\text{m})^2$ scan taken at 2 Hz. A line scan, in red, shows the thickness of the primary lipid components. The gel phase (predominantly DPPC) is nominally 5.2 nm, the fluid phase (predominantly POPG) 3.6 nm, and the surfactant proteins and palmitic acid features are 5.6 nm. All heights are relative to the underlying mica substrate.

Figure 3.

Structures of the two primary lipid components in Survanta. The transition temperature of the DPPC is $\sim 41^\circ\text{C}$, putting it in the gel phase under experimental conditions. The choline terminal group creates a zwitterionic lipid. The unsaturated acyl chain in POPG leads to a significantly lower transition temperature, 2°C , as well as a roughly 1.5 nm difference in bilayer height. The glycerol termination results in an anionic lipid.

Figure 4.

This figure shows the time progression of defects caused by 90 nM G5-PAMAM dendrimer. Image A is ~2 min before the addition of dendrimer, Images B, C, and D are 2, 4, and 6 min following the addition of dendrimer. The blue arrows in Image C show expansion of existing defects by the dendrimer, predominantly in Domain B. Purple arrows in Image D show new defect formation. The teal arrow in Image D shows the accumulation of material around a defect. Red boxes highlight linescan regions used for Figure 8.

Figure 5.

This figure shows the time progression of defects caused by 110 nM G7-PAMAM dendrimer. Image A is ~4 min before the addition of dendrimer, Images B, C, D, and E are 8, 12, 16, and 20 min following the addition of dendrimer, respectively. The blue arrows in Images C and D again show expansion of existing defects. Teal arrows in Images C and D indicate an accumulation of material that precedes lipid removal. Red boxes show regions used for line scans for Figure 8.

Figure 6.

In this figure, lipid removal by 110 nM G7-Amine is illustrated. All images, including the line scan, share a 7 nm height scale. Images A and B are ~2 min apart. Red boxes highlight the regions for Images C and D, which have been magnified by a factor of 4. Teal arrows indicate regions of accumulated dendrimer that precede lipid removal. No dendrimers are observed on mica surface immediately following lipid removal. Image E is an image of G5-Amine dendrimer on mica at the same imaging speeds and scan size as before. 40 μ L of 1.1 mM G5-Amine was spin coated onto freshly cleaved mica preceding tapping mode imaging in water. The observed streaking is consistent with work done by Betley,²² which found low pH $\approx$ 1 was needed to abolish streaking and fully adhere the dendrimers to the mica. The image has been cropped to the same scale as images C and D. The gray line labeled “1” indicates where a linescan was taken to determine dendrimer size. Bottom left: line scan across the dendrimers. They are between 2 and 3 nm in height and 15 nm in width. The width of the peaks shown in the linescan is $\sim$ 36 nm. This is due to the radius of curvature of the tip (20 nm).

Figure 7.

This figure shows the phase image during Figure 5E. The phase difference measures the phase lag between the oscillation driving the cantilever and the readout at the split photo diode. Hard, perfectly elastic materials, like mica, appear black, while softer and stickier surfaces will have a larger phase lag and will thus be lighter. An advantage of phase imaging is that it can be used to differentiate materials. The phase lag in Domain A is significantly lower than in Domain B, presumably due to the gel nature of Domain A. More importantly, the regions corresponding to accumulated dendrimer show an additional phase lag from the surrounding lipid in both Domain A and Domain B. This suggests that the dendrimer is either sitting on top of the lipid, or partially intercalated into the hydrophilic region yet still somewhat exposed. On the right, are images from a molecular dynamics simulation showing the interaction of a G3 PAMAM dendrimer with a DMPC bilayer. In Image A the dendrimer is effectively not interacting with the bilayer. In Image B, the dendrimer is interacting with a gel DMPC bilayer (similar to domain A). In Image C the dendrimer is interacting with a fluid DMPC bilayer.

Figure 8.

Highlighted regions in Figures 4 and 5 were selected because they showed raised edges preceding lipid removal. Between 5 and 18 line scans were taken across each region and averaged to generate the figure. Each line scan has been positioned so the step from lipid to the accumulated material occurs at the same position. In each case, the height of the reference lipid has been set to 0. For G5-Domain A, G7-Domain A, and G7-Domain B the reference lipid is on the right and the accumulated material on the left. For G5-Domain B the scan goes from a hole at the edge of the accumulated material across the step and down to the reference lipid. The line scans were taken parallel to the fast scan axis of each AFM image. Error bars are the standard error at each location (pixel). For G5 interacting with Domain A, and G7 interacting with both Domain A and Domain B a significant accumulation of material greater than 0.5 nm indicates this is not a phase transition.

Table 1
Accumulation Height at the Leading Edge of Each Material^a

	Domain B	Domain A
G5	0.11 ± 0.08 nm	0.85 ± 0.01 nm
G7	0.63 ± 0.01 nm	0.84 ± 0.01 nm

^a Statistically identical heights in the gel phase for G5 and G7, yet much smaller values for Domain B than Domain A, indicate that the ability of the dendrimer to reach out and interact with the lipid and or penetrate the bilayer is mediated primarily by the fluidity of the layer and not electrostatic interactions. The stated error for G5-Domain B is from the standard error of the data set. For the rest the calculated error was below significance for both instruments so the error was rounded to the nearest significant figure.