Published in final edited form as:
Langmuir. 2005 September 13; 21(19): 8588–8590.

Synthetic and Natural Polycationic Polymer Nanoparticles Interact Selectively with Fluid-Phase Domains of DMPC Lipid Bilayers

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Abstract

Polycationic polymers are known to disrupt lipid bilayers. In this letter, we report the dependence of this disruption on the lipid structural phase. DMPC bilayers are exposed to two polycationic polymeric nanoparticles, PAMAM dendrimers and MSI-78. We find that regions of the bilayer that are in the gel phase are unaffected by the presence of polymers, whereas the liquid phase is disrupted.

Polycationic polymers are used extensively for the transport of material across cell membranes. Synthetic polymers commonly employed include linear macromolecules such as polyethyleneimine (PEI), poly-L-lysine (PLL), diethylaminoethyl-dextran (DEAE-dextran), and branched polymers such as poly(amidoamine) (PAMAM) dendrimers.[1-3] Interestingly, several classes of natural polycationic polymers appear to play a similar role, albeit by a different mechanism, including the cell-penetrating peptides or CPPs.[4-6] The details of cellular internalization of the proteins and polymers are only partially understood and have been the subject of considerable debate in the literature.[7-12]

For the synthetic polycationic polymers PEI, PLL, DEAE-dextran, and PAMAM dendrimers, it has been reported that uptake substantially decreases or ceases upon cooling the cell to ~6 °C.[8,10] This energy dependence has been interpreted as supporting an ATP-dependent endocytosis mechanism. Recently, an alternative hypothesis for polycationic polymer internalization has been proposed that is also temperature-dependent—the formation of nanoscale holes.[8,13] For the case of CPPs, both temperature-dependent endocytosis processes and temperature-independent membrane translocation processes have been proposed.[9,11,14-16]

To explore the possible role of membrane phase changes upon the interaction of these materials with the cell membrane, we have employed a model system consisting of a supported 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) lipid bilayer on mica. We employed this

single-component model so that we could focus solely on the issue of phase change and not have additional complications arising from changes in the structure and/ or organization of additional components in the membrane as a function of temperature. The DMPC lipid was chosen because the phosphocholine headgroup is a common component in mammalian lipids. The myristoyl (C14) alkyl chains give this supported lipid bilayer on mica a broad gel to liquid-crystalline phase-transition temperature centered at 28.5 °C.[17] At this temperature, the sample is roughly half gel phase and half liquid phase. The two phases are easily differentiated by atomic force microscopy (AFM) allowing direct comparison of the interactions of the polycationic materials with the two different membrane phases.

In this letter, we report AFM experiments on model membranes consisting of supported DMPC bilayers and de-Paked ^{2}H quadrupole coupling nuclear magnetic resonance (NMR) experiments on DMPC lipid multilayers. Lipid bilayers can exist in several structural states. In this study, we are concerned with two such states: a hightemperature liquid-crystalline fluid phase and a lowtemperature gel phase. These are commonly referred to as L_α and $L_{\beta'}$* phases, respectively.[18] Because the two phases differ in the thickness of the lipid bilayer, temperature-induced phase changes in bilayers can be observed by AFM.[17,19] Additionally, the difference in the acyl chain order can be measured using NMR experiments. For example, the magnitude of the ^{2}H quadrupole splitting of the methylene units of DMPC provides a sensitive probe of the membrane phase.[26,27]

The central observation reported in this letter is the strong dependence on the lipid phase that controls the interaction between polycationic polymeric nanoparticles and the lipid bilayers. Specifically, it is found that $L_{\beta'}$* phase domains in the bilayers are unaffected when exposed to the nanoparticles whereas L_α phase domains are strongly disrupted or destroyed.

The synthetic polycationic polymer studied was generation 7 PAMAM dendrimer (G7-NH_2).[20] These are spherical branched macromolecules 8 nm in diameter[21] with amine-terminated branch ends. PAMAM samples used in this study were synthesized at the Center for Biologic Nanotechnology, University of Michigan, Ann Arbor, MI. Because of protonation at pH <7, they carry a net positive charge when dissolved in water.[22] Recent experiments have shown that PAMAM dendrimers are able to form nanoscale holes in the L_α phase lipid bilayers and to permeabilize cell membranes.[7,8,13] Studies indicate that dendrimers remove lipid molecules from the bilayer membrane leaving behind a defect.

To observe membrane disruption at high spatial resolution (~10 nm) and as a function of time, AFM images of supported DMPC bilayers before and after the addition of G7-NH_2 were recorded. Lipid bilayers were formed on mica by vesicle fusion and imaged with a Multimode AFM (Digital Instruments, Veeco Metrology, Santa Barbara, CA) in noncontact mode as described previously.[8,13] Before placing the sample in the AFM, the lipid bilayer was rinsed with cold water or refrigerated for about 15 min. In this manner, bilayer samples with two coexisting phases were obtained (Figure 1a). Over the course of 20-30 min at normal laboratory conditions, the sample temperature increased until the transition from the $L_{\beta'}$* to L_α phase was complete. At a point where both L_β* and L_α phases were apparent in the AFM images, approximately 30 μL of the aqueous polymer solution was injected into the sample volume. All experiments were performed in DI water at pH 6.

In the case of G7 PAMAM dendrimers (Figure 1), the polymers diffused to the substrate within 2 min, resulting in the formation of holes in the lipid bilayer as reported earlier.[13] Although 60-70% of the scanned bilayer area was in the $L_{\beta'}$* state when the dendrimers were added, no dendrimer activity was observed in this domain. In contrast, the L_α phase is disrupted within a few minutes of exposure to the dendrimers. These data show two prominent differences in the observations previously made when the entire bilayer was in the L_α phase.[13] During these

mixed-phase experiments, hole formation proceeded more slowly, over a period of 2-20 min, as opposed to within the first 2-5 min for the single L_α phase samples. Additionally, for the lower-temperature mixed-phase experiment, aggregates of dendrimers or dendrimer—lipid assemblies were occasionally observed on the bilayer (seen as white spots in images 1b and c). After a short period of time, these aggregates lifted off the surface, leaving behind bilayer defects (image 1d).

The second polycationic polymer employed for this study was MSI-78 (Pexiganan), a 22-residue peptide with the amino acid sequence G-I-G-K-F-L-K-K-A-K-K-F-G-K-A-F-V-K-I-L-K-K-NH_2. It is an analogue of the magainin2 family, a class of naturally occurring antimicrobial peptides found in frog skin. The MSI-78 sample was designed and synthesized by Genaera Corporation (Philadelphia, PA). At low peptide/lipid molar ratios, these peptides adsorb to lipid bilayers with their amphipathic α-helical axis parallel to the membrane surface.[23,24] Although MSI-78 is known to have a different mechanism of membrane disruption,[23-25] we find that it also selectively attacks only fluid L_α phase regions of membranes.

Figure 2 shows that peptides induce membrane thinning only in the L_α phase regions of the lipid bilayer. In this Figure, the topographical data is shown on the left, and the corresponding phase contrast, on the right. Note that the term “phase” in this context is not to be confused with the structural phase of a solid or liquid but instead is related to the phase lag between the driving force and the oscillating AFM cantilever. This phase lag is the result of energy dissipation during tip—sample interaction and depends, among other parameters, on the elastic modulus of the sample surface. The phase lag signal clearly shows the boundaries between $L_{\beta'}$* and L_α regions of the bilayer. The regions where the peptide adsorbs appear black in both cases.

Previous NMR studies have shown that MSI-78 induces positive curvature strain on lipid bilayers and disrupts via toroidal pore formation.[25] In particular, the magnitude of the ^2H quadrupole splitting of the methylene units can be used to assess the degree of peptide-induced disorder in the lipid acyl chains and therefore is a sensitive indicator of MSI-78 binding to the headgroups.[26,27] To perform this experiment, multilamellar vesicles (MLVs) were prepared by mixing 65 mg of DMPC-d_{54} with 3 mol % MSI-78 in 2:1 $CHCl_3$/MeOH. The peptide/lipid sample was dried under a stream of nitrogen and then under vacuum overnight to completely remove any residual solvent. ^2H-depleted water (60 wt %) was added to the dry peptide/lipid mixture, vortexed, and subjected to 10 freeze—thaw cycles. NMR experiments were performed on a Chemagnetics/Varian Infinity 400 MHz solid-state NMR spectrometer operating with a resonance frequency of 61.424 MHz for ^2H nuclei. A Chemagnetics temperaturecontroller unit was used to maintain the sample temperature, and the sample was equilibrated for at least 30 min before starting each experiment.

^2H NMR spectra of DMPC bilayers containing 3 mol % MSI-78 at two different temperatures are given in Figure 3. Peptide binding causes membrane thinning, which is consistent with an increase in the disorder of lipid acyl chains. This effect is shown in Figure 3 as a decrease in the ^2H quadrupole splitting of the methylene units of DMPC at 30 °C where the bilayers are in the fluid lamellar L_α phase. However, the peptide does not decrease the quadrupole splitting when the bilayers are in the gel $L_{\beta'}$* phase, indicating that peptide-induced disorder is negligible at this temperature. Thus, the NMR and AFM data are in excellent agreement with the conclusion that MSI-78 selectively induces disorder in the L_α phase membrane but not in the $L_{\beta'}$* phase membrane.

In summary, two very different classes of polycationic polymeric nanoparticles are observed to interact selectively with the liquid-crystalline fluid L_α phase lipid bilayer and not with the

gel $L_{\beta'}$* phase lipid bilayer. This study suggests that the membrane phase should be explicitly considered when employing low temperature or chemical species that can influence the membrane structural phase for studies of cellular uptake of these materials. The data presented here indicates that perturbations to the cell that induce partial or complete conversion of the plasma membrane to the gel phase would be predicted to inhibit or prevent polycationic polymer nanoparticle interactions with the membrane. This would be expected to have a significant impact upon the transport of these materials across the membrane (PAMAM dendrimers) or the ability of the materials to forms pores (MSI-78).

Acknowledgment

This project has been funded with federal funds from the National Cancer Institute, National Institutes of Health (NIH), under contract no. NOI-CO-97111 (to M.M.B.H. and B.G.O.) and by NIH grant AI054515 (to A.R.). We thank Dr. Lee Maloy (Genaera Pharmaceuticals) for providing us with unlabeled MSI-78 peptide.

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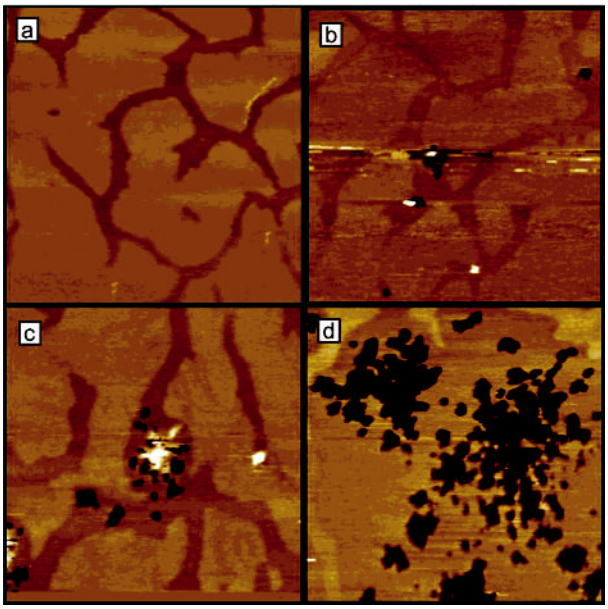

Figure 1.

AFM height images of the supported DMPC bilayer during phase transition before and after the addition of 25 nM G7 PAMAM dendrimers. (a) Before adding dendrimers. The height difference between the gel $L_{\beta'}$* phase (lighter shade) and the liquid L_{α} phase (darker shade) is approximately 0.5 nm.[14,15] (b) 3, (c) 8, and (d) 17 min after adding dendrimers. Defects (black areas) caused by dendrimers are approximately 5 nm deep. Scan size 1 μm, color height scale 0-5 nm.

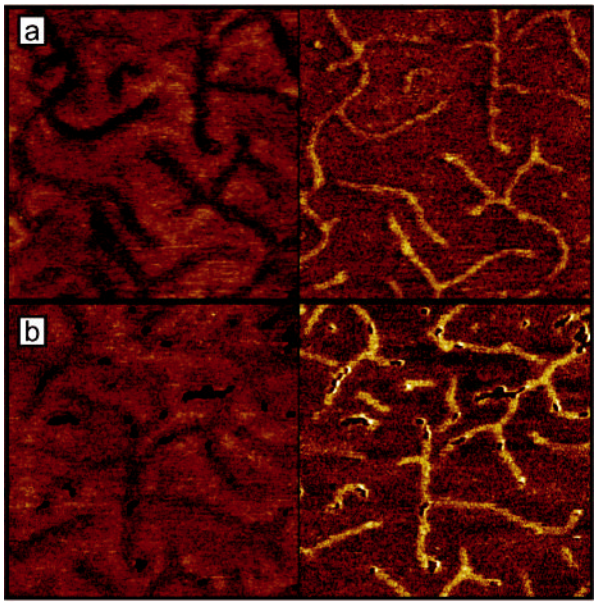

Figure 2.
AFM height images (left) and corresponding phaselag images (right) of the DMPC bilayer during phase transition (a) before and (b) after adding 1 μM MSI-78. Scan size 1 μm, color scale of height images 0—5 nm. Phase contrast shows very clearly that the peptide is adsorbed selectively to the L_α regions of the bilayer.

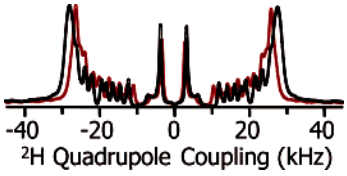

Figure 3.

De-Paked ^{2}H quadrupole coupling NMR spectra of d_{54}-DMPC bilayers containing 3 mol % MSI-78 at 25 °C (black) and 30 °C (red). ^{2}H quadrupole coupling spectra were obtained using a quadrupole-echo sequence (90°-τ-90°- τ-acquire) with a 90° pulse length of 3.0 μs, a spectral width of 100 kHz, 15 000 scans, and a recycle delay of 2.5 s. The spectra were processed using Spinsight (Chemagnetics/Varian) software on a Sun Sparc workstation and de-Paked using Matlab software. Note that DMPC bilayers are still in the gel L_β* phase in the presence of MSI-78 at 25 °C because peptide binding slightly increases the main phase-transition temperature of the lipid.