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Molecular Dynamics Simulation of PEGylated Bilayer Interacting with Salt Ions: A Model of the Liposome Surface in the Bloodstream

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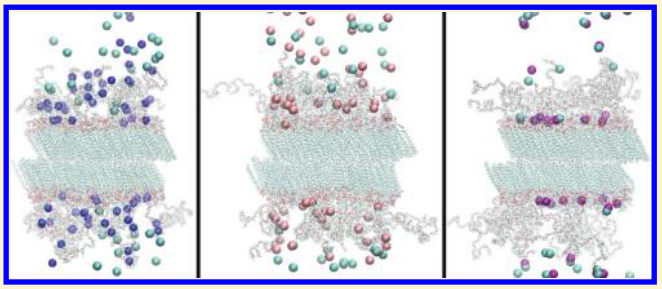
ABSTRACT: PEGylation is an effective mechanism to prolong the bloodstream lifetime, and thus efficacy, of drug delivery liposomes. The mechanism through which poly(ethylene glycol) (PEG) increases bloodstream lifetime is, however, not completely understood. The interaction with salt ions found in the bloodstream is known to play a role in this. We have used all-atom molecular dynamics simulation to study the effect of PEGylated lipid density, salt concentration, and the interaction with KCl and $CaCl_2$ salts in addition to NaCl. Increasing the PEGylated lipid concentration in the formulation from 1:18 to 1:9 molar density decreased the extent to which the Cl^- ions penetrated the PEG layer, thus causing the PEG layer to become effectively positively charged. The interaction of the PEG with the K^+ ions was weaker than for the Na^+ ions, and nonexistent for the Ca^{2+} ions. This work expands on our previous work where we studied the gel and liquid crystalline membranes in physiological salt concentration. Our results provide both an explanation for the experimental observation that PEGylation inhibits calcium-induced liposome fusion and further insight into the mechanisms through which PEG may inhibit uptake of the liposome by the reticuloendothilial system (RES).



INTRODUCTION

Liposomes have been used as carrying devices in drug delivery since the 1970s,[1] and continue to be a promising avenue for drug delivery.[2,3] One of the most important developments in drug delivery liposome (DDL) design is "PEGylation": including phospholipids with an attached poly(ethylene glycol) (PEG) with a molecular weight in the range 1−5 kDa into the DDL formulation at a molar fraction in the range of 3−15%. This results in the PEG polymer forming an outer shell of the DDL, with two beneficial effects: (1) the PEG forms a "stealth sheath", a protective polymer coating[4] that reduces uptake of the DDLs by the reticuloendothelial system (RES)[5] and (2) enhanced take up to tumor vasculature through the enhanced permeability and retention (EPR) effect.[6]

In terms of reducing RES uptake, PEGylation is extremely successful; PEGylation increases bloodstream circulation from ∼1 h to the range of 1−2 days.[7] There is, however, still considerable room for improvement; red blood cells, blood platelets, and some antibodies have a blood plasma circulation time in the range of months. Toward this end, several alternative protective polymer coatings to PEG are currently being investigated.[8] A rational approach to improving the design of the protective polymer coating requires an understanding of the mechanism through which PEG impedes RES uptake. The current picture we have of this is, however, unclear.

The first step to the removal of foreign particles by the RES is opsonization,[5] where a set of bloodstream proteins adhere to the outside of particles and identify them so they can be eliminated by macrophages. The interaction between DDLs and bloodstream proteins has been the subject of many studies.[7] The results of these studies are, however, unclear: while some studies indicate that PEGylation inhibits protein adhesion,[9] other studies have found this not to be the case.[10] Alternative mechanisms that have been proposed include inhibition of liposome fusion and acting as a direct steric barrier against macrophages.[10] All of these mechanisms are clearly mediated by the properties of the surface of the DDL. Particularly important is the surface charge, in part resulting from interaction with bloodstream ions. Surface charge is known to play a role in mediating opsonization.[5]

In a previous work,[11] we have shown that the previously accepted picture of PEG as a generic neutral hydrophilic polymer located entirely outside the lipid bilayer is incorrect. To model the PEGylated liposome surface, we performed molecular dynamics (MD) simulations of a PEGylated bilayer with 1:9 molar fraction, in both the liquid crystalline and gel states at physiological NaCl concentration. We found that PEG strongly associates with Na^+ and that in the liquid crystalline membrane, but not the gel membrane, some of the PEG polymers locate to the lipid core of the membrane. As we noted in that publication, neither of these results should be surprising since it is well-known that PEG both acts as a polymer

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Table 1. Number of Each Molecule in Each System

no.	state	salt concn (mM)	DLPC	DLPE-PEG	DSPC	DSPE-PEG	Na^+	K^+	Ca^2+	Cl^-	water
1	LC	0	464	48	0	0	48	0	0	0	72851
2	LC	125	464	48	0	0	189	0	0	141	72642
3	gel	0	0	0	256	32	32	0	0	0	25896
4	gel	125	0	0	256	32	64	0	0	32	25885
5	gel	125	0	0	256	32	0	64	0	32	25885
6	gel	125	0	0	256	32	0	0	32	32	25885
7	gel	125	0	0	272	16	48	0	0	32	25885

electrolyte, associating with cations,[12] and is soluble in a wide variety of both polar and nonpolar solvents.[13] As a result of these two effects, we found that, at 10% molar concentration of PEGylated lipid, in the gel state the PEG layer has an effective positive charge due to the Cl^- ions being expelled from the tighter PEG layer structure, but for the liquid crystalline case the PEG layer is effectively neutral due to the Cl^- ions being able to locate in water pockets within the PEG layer. The fact that the PEG polymer penetrates into the lipid core of the liquid crystalline, but not the gel membrane, can be attributed to the looser structure of the liquid crystalline membrane.

We build on our previously published work by studying the effect of (1) removing the salt, leaving only counterions, (2) lowering the PEGylated lipid molar fraction from 1:9 to 1:18, and (3) investigating the interaction with two other salts that are biologically relevant: KCl and $CaCl_2$. In our system, the PEGylated membrane solvated in salt solution, the cations, in this study Na^+, K^+, and Ca^{2+}, have a choice of four environments: (1) free in the water, (2) bonded to the Cl^- anions, (3) bonded to the PEG oxygens, or (4) bonded to the lipid headgroup oxygens. In our previous work,[11] we found the Na^+ ions are predominantly bonded to the PEG oxygens but also to a lesser degree to the headgroup oxygens. The determination of how the Na^+, K^+, and Ca^{2+} ions differ in how they partition between these four environments is one of the goals of this study.

We found that (1) the cations in the PEG layer distribute further out within this layer, and the PEG layer itself expands when salt is present and, for the liquid crystalline membrane, the presence of salt increases the depth to which the PEG penetrates the membrane interior; (2) the PEG association with K^+ ions is weaker than with Na^+ ions and Ca^{2+} ions show no interaction with PEG and thus do not locate to the PEG layer; and (3) when the PEG density is reduced to a 1:18 molar fraction, we see the same effect in the gel membrane as we did in the liquid crystalline membrane at 1:9 molar fraction: the less dense PEG layer is able to incorporate water pockets containing the Cl^- ions, thus increasing the extent to which Cl^- can penetrate the PEG layer, reducing the effective charge of the PEG layer, and thus lowering the electrostatic potential at the PEG layer.

METHODS

Systems Simulated. In this study, we simulated seven separate membrane systems, all of PEGylated membranes; the number of each type of molecule/ion in each system is shown in Table 1. Membranes in both gel and liquid crystalline states were used to study the PEGylated membrane structure in absence of salt, with only Na^+ counterions to balance the negative charge held by each of the PEGylated lipids present. The gel membranes were composed of a mixture of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine-poly(ethylene glycol)-2000 ($DSPE\text{-}PEG_{2000}$) lipids. The liquid crystalline membrane was composed of 1,2-dilinoleoyl-*sn*-glycero-3-phosphatidylcholine (DLPC) and DLPE-PEG lipids. Gel membranes were simulated with NaCl at physiological salt concentration (125 mM) and KCl and $CaCl_2$ set to the same ionic strength (concentration 125 mM for KCl and 62.5 mM for $CaCl_2$) for comparison, with a PEGylated lipid fraction of 1:9. A gel membrane with physiological salt concentration was also simulated with a PEGylated lipid fraction of 1:18. All simulations were carried out at a constant pressure of 1 bar and physiological temperature (310 K).

Construction of the Seven Systems. All seven systems simulated were constructed from the final configurations of the simulation trajectories created in our previous study. For the two systems without salt, the salt ions removed were replaced by water molecules. For the system with $CaCl_2$ as the salt, the excess cations were also removed in this fashion. For the system with the PEGylated lipid fraction reduced from 1:9 to 1:18, the DSPE-PEG lipids were replaced with DSPC lipids. The empty space left by the removed PEGs was allowed to fill with water as the simulation was carried out at constant pressure to equilibrate the system.

Molecular Model Parametrization. All parametrization details regarding membrane lipid molecules were carried out in the same fashion as our previous study[11] that involved the same set of lipid molecules. For all molecules and ions in our simulation, the all-atom OPLS force field[14] was used as described in our previous studies.[11,15−20] With respect to partial charges, for the case of the PC headgroups they were taken from Takaoka et al.[21] as derived in compliance with the OPLS methodology, and in all other cases they were taken from the OPLS parameter set. The details of how the charge groups have been defined can be seen in our previous work.[17] For water, the TIP3P model, compatible with the OPLS-AA parametrization,[22] was used.

Molecular Dynamics Simulation Parameters. Periodic boundary conditions with the usual minimum image convention were used in all three directions. To preserve the covalent bond lengths, the linear constraint solver (LINCS) algorithm[23] was used. The time step was set to 2 fs. The temperature and pressure were controlled using the Nosé−Hoover[24,25] and Parrinello−Rahman[26] methods, respectively. The temperatures of the solute and solvent were controlled independently. For pressure, we used a semi-isotropic control. The Lennard-Jones interactions were cut off at 1.0 nm, and for the electrostatic interactions we employed the particle mesh Ewald method.[27]

System Size and Equilibration Time. For the gel membranes the system sizes were *x* and *y* components, parallel to membrane, of 8 nm, and *z* component, perpendicular to membrane, of 18 nm. For the liquid crystalline systems the *x*, *y*,

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and z dimensions were 13.5, 13.5, and 17 nm, respectively. In our previous publication,[11] we determined that this system size is large enough to avoid finite size issues. All simulations were carried out for 100 ns longer than the equilibration time so that we have a 100 ns trajectory for analysis for each system. The equilibration time is determined from the time needed for ions to adsorb to the polymer layer and the membrane interface. In quantitative terms this corresponds to the number of ions measured to be in each of the four possible environments (see Introduction) to reach an equilibrium value. In our previous research[11,19] we determined that concerning the properties that we are studying, this is the most slowly relaxing variable. For all systems with NaCl, this was seen to occur after 100 ns, and for the systems with KCl and $CaCl_2$, 160 ns.

Simulation and Analysis Software Used. All molecular dynamics simulations were carried out using the GroMACS simulation package, version 4.5.[28] Visualization of the trajectories was performed using Visual Molecular Dynamics (VMD).[29] Some of our analysis was performed using perl and Fortran95 scripts that we wrote ourselves.

Analysis Protocol. Five separate forms of analysis were performed on our simulated systems. First, we visualized the systems to obtain an intuitive picture of the systems. Next, we determined the number of cations in each of the four possible environments in each of the systems at each time step. Since ion binding is the most slowly relaxing variable regarding the properties we intend to study, the convergence of these quantities was used to determine the effective equilibration time for each system. For all systems we simulated 100 ns beyond the equilibration time and determined the number of ions in each of the four environments from the average value over this time. For the ions bound to oxygens or anions we defined binding using the same protocol used in our previous publications.[11,19,30] We then measured the mass density profile perpendicular to the membrane normal for PEG, anions, cations, and phosphate headgroups, for each system. After this, we measured the radial distribution function (RDF) between cation and headgroup oxygens, PEG oxygens, and anions, for each of the three equivalent salt systems. Finally, we measured the electrostatic potential along the membrane normal for each system. This involves first calculating the charge density, then integrating this twice over the Z coordinate. This calculation was also performed in our previous work[19] using the same procedure.

RESULTS

PEGylated Membrane Structure in Absence of Salt. Simulations were carried out of both gel (DSPC) and liquid crystalline (DLPC) membranes with PEGylated lipids at molar density 1:9 with (1) NaCl at physiological concentration (125 nm) and (2) with only Na^+ counterions. For each PEGylated lipid, a cation (counterion) is needed since PEGylation results in a net negative charge of the lipid of −1. The results for the mass density profile along the membrane normal are shown in Figure 1. When the salt is removed, the same two peaks in the mass density profile for Na^+, (1) at the membrane headgroup and (2) within the PEG layer, can be be observed in all cases. These correspond to Na^+ ions bound to the lipid headgroups and Na^+ ions associating with the PEG polymer respectively as seen our previous published work.[11]

The distribution of Na^+ ions within the PEG layer is shifted closer to the membrane side of the PEG layer for both gel and liquid crystalline systems when the salt is removed. For the case

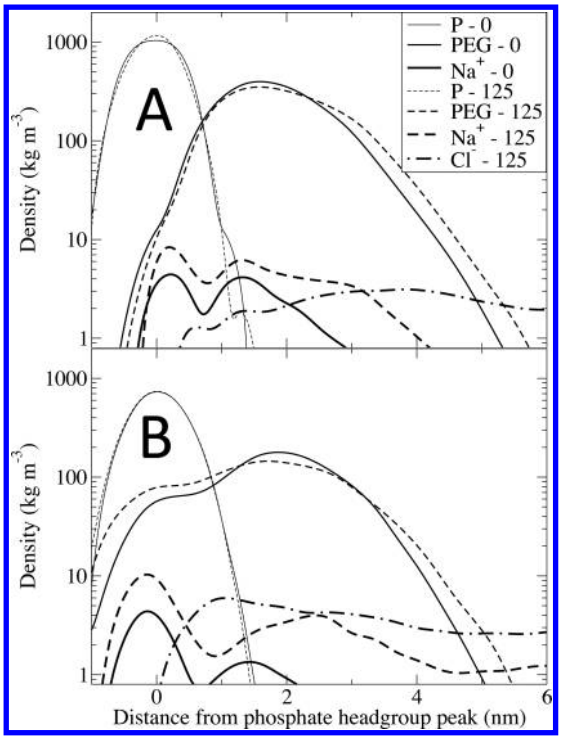

Figure 1. Mass density profile along the membrane normal of membrane headgroup P, PEG, Na^+ cations, and Cl^- anions for (a) the gel membrane (DSPC) and (b) the liquid crystalline membrane (DLPC), comparing the case of physiological salt concentration (125 mM) and only Na^+ counterions. We see that the presence of the Cl^- anions shifts the Na^+ in the PEG layer further out into the layer. The presence of salt can also be seen to expand the PEG layer slightly, for the liquid crystalline case also increasing the depth of its penetration into the membrane interior.

of the liquid crystalline membrane, a portion of the Na^+ ions are floating free in the solution, outside the PEG layer, only when salt is present. Additionally, the presence of salt can be seen to slightly expand the PEG layer of the membrane. For the liquid crystalline membrane, the presence of salt also increases the depth to which the PEG penetrates into the membrane interior. Results for the electrostatic potential (not shown) indicate, as expected, that when the salt is removed the potential peak in the PEG region is decreased.

Effect of Alteration of Formulation Density of PEGylated Lipids. Simulations were carried out of the PEGylated membrane in physiological concentration of NaCl for PEGylated membranes at two separate molar concentrations of PEGylated lipid in the membrane formulation: 1:9 and 1:18. From the mass density profile along the membrane normal (Figure 2A), we see that the Na^+ cation distribution is unchanged. A striking effect can, however, be seen on the profile of the Cl^- ions. The extent to which the Cl^- ions penetrate the PEG layer is significantly greater in the less dense 1:18 formulation than in the 1:9 formulation. The same effect was also observed in our previous study[11] for the liquid crystalline membrane at 1:9 formulation density where the effective density of the PEG layer is also reduced. Visualizing our system, this effect is demonstrated, as shown in Figure 3. The results for the electrostatic potential, shown in Figure 2B, indicates that the electrostatic potential peak at the PEG region is significantly greater for the 1:9 system. This is exactly the result we would expect, as decreased penetration of Cl^- into the

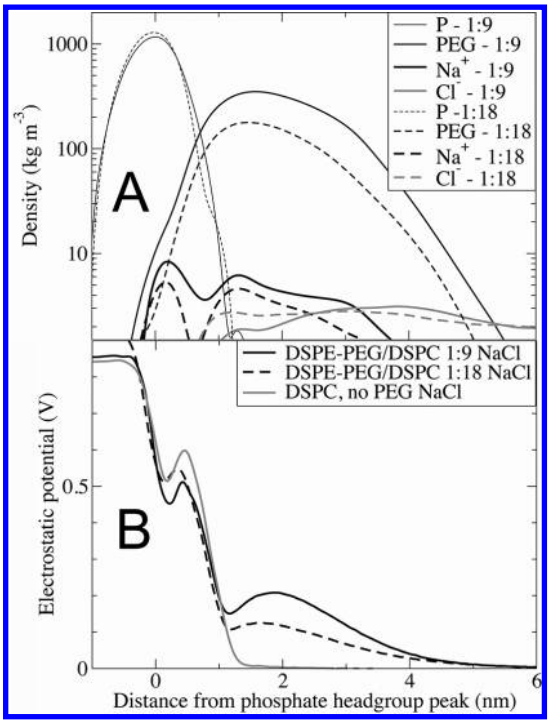

Figure 2. Comparison of different PEG densities: (A) mass density profile along the membrane normal of membrane headgroup P, PEG, Na^+ cations, and Cl^- anions for PEGylated bilayer with PEGylated membranes of 1:9 and 1:18 molar fraction of PEGylated lipids. We see that the Cl^- ions penetrate considerably deeper into the PEG layer for the lower formulation density than for the higher formulation density. (B) Electrostatic potential along the membrane normal shows that the potential peak in the PEG region is significantly lower for the membrane with lower formulation density of PEG.

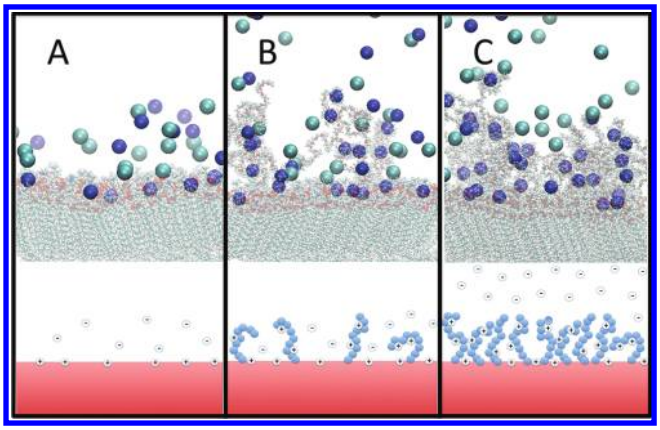

Figure 3. Visualization of gel (DSPC) membrane in physiological salt concentration (125 mM): (A) without PEG (result from previous publication), (B) at 1:18 molar density PEGylated lipids, and (C) 1:9 molar density PEGylated lipids. We see the discussed mechanism whereby the Cl^- ions are able to penetrate deeper into the PEG layer for the case of the lower PEG formulation.

PEG layer will result in an increase in the strength of the charge double layer at the PEG surface.

Interaction of Membrane with Salt Ions for KCl and $CaCl_2$ in Comparison to the Result for NaCl. Simulations were carried out for gel membrane systems with NaCl at physiological concentration (125 mM) and 1:9 molar concentration of PEGylated lipid along with identical lipid systems solvated in the same ionic strength for KCl and $CaCl_2$: concentration 125 mM for KCl and 62.5 mM for $CaCl_2$. The interaction of the three salts with the PEGylated membrane are very different as can be seen from our visualization of the simulation, shown in Figure 4. We see that the Na^+ cations

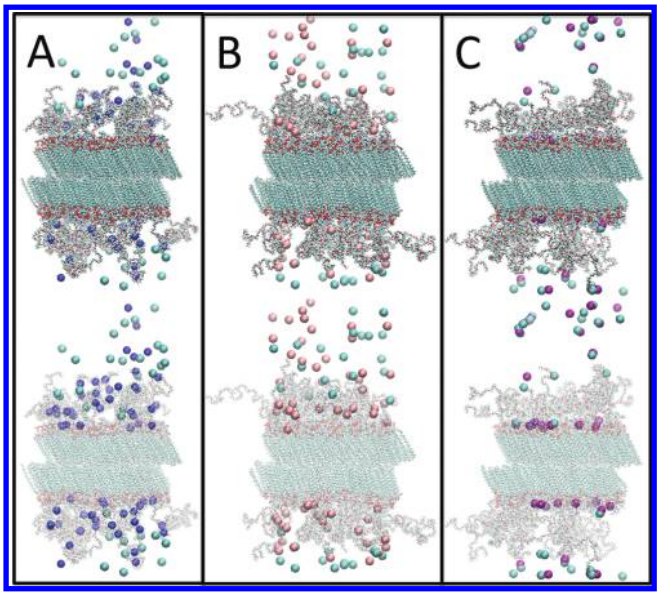

Figure 4. Visualization of the PEGylated mebranes at 1:9 PEGylated lipid formulation density with different salts all at 125 mM concentration: (A) NaCl, (B) KCl, and (C) $CaCl_2$. Note the absence of Ca^{2+} ions in the PEG layer and their close association with both the lipid headgroups and the Cl^- anions.

strongly interact with the PEG, as we have seen in our previous study.[11] The K^+ ions can also be found in the PEG layer, though we see a larger portion of them remaining in solution outside the membrane than the case for the Na^+ cations. The Ca^{2+} cations do not collocate to the PEG layer at all and can be seen bound to the lipid headgroups and in solution. The binding to Cl^- ions is particularly strong, so strong in fact that some of the Cl^- ions are brought to the lipid headgroups with the Ca^{2+} cations.

Our results for the mass density profile for all ions in comparison (Figure 5A) demonstrate these results: both the K^+ and Ca^{2+} cations show a significant constant density outside the PEG membrane in the bulk fluid; however, this is not the case for the Na^+ cations. No mass density peak in the PEG region can be seen for Ca^{2+} ions, and a small mass density peak for the Cl^- ions can be seen at the position of the membrane headgroups for the $CaCl_2$ system. Our results for the electrostatic potential along the membrane normal, Figure 5B, indicate that the potential peak in the PEG region is decreased for the KCl and $CaCl_2$ systems relative to the NaCl system, and the electrostatic potential peak at the membrane headgroup is weaker for the KCl system than for the NaCl and $CaCl_2$ systems.

We then calculated the radial distribution function (RDF) (Figure 6) for the three cations with the three relevant electronegative sites in the system: (A) the Cl^- anions, (B) the PEG oxygens, and (C) the three headgroup oxygens. We found no interaction between the PEG headgroup oxygens and the Ca^{2+}, and the association with the Cl^- anions is much stronger for Ca^{2+} than for the two monovalent cations. The larger effective size of the K^+ ions is evident and, in comparison to the Na^+ ions, the binding to all three electronegative sites is equally

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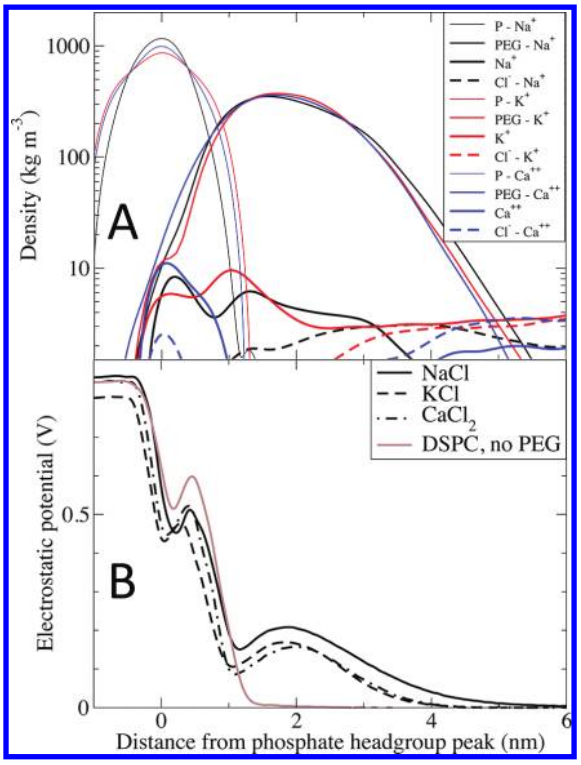

Figure 5. Comparison of the different ions. (A) Mass density profile along the membrane normal of membrane headgroup P, PEG, Na^+ cations, and Cl^- anions for PEGylated bilayer with formulation density of 1:9 and different salts (NaCl, KCl and $CaCl_2$) all at 125 mM concentration. Note (1) the absence of Ca^{2+} ions in the PEG layer, (2) the K^+ and Ca^{2+} ions but not the Na^+ ions can be found in the bulk solution, and (3) some Cl^- ions that have been brought to the lipid headgroups by their strong attraction to the Ca^{2+} ions. (B) Electrostatic potential along the membrane normal shows the potential peak due to the PEG layer to be stronger in the presence of NaCl than KCl or $CaCl_2$.

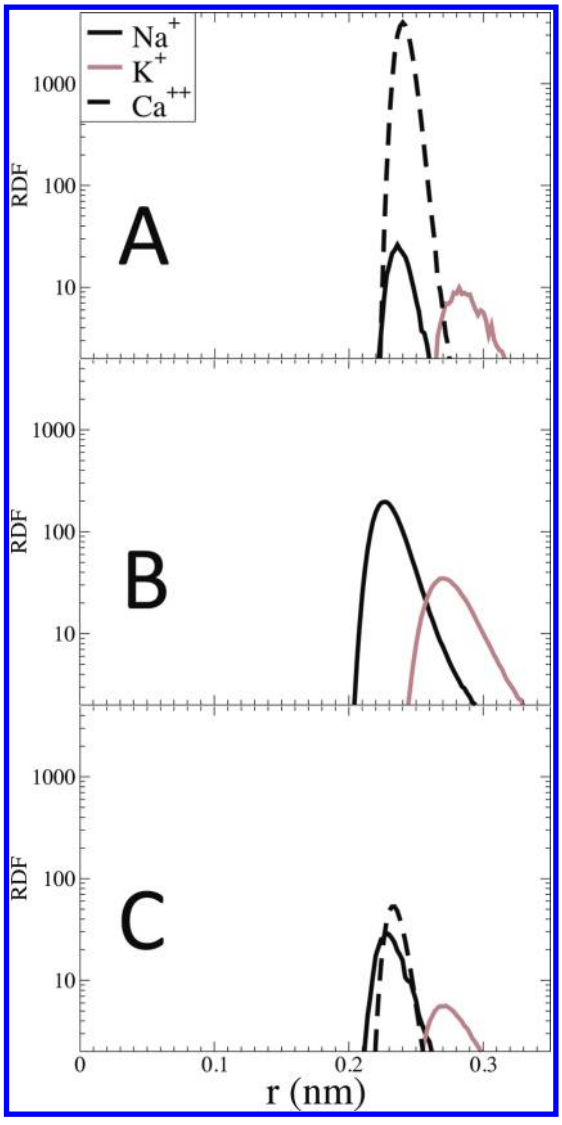

Figure 6. Radial distribution function of the three cations with (A) the Cl^- anions, (B) the PEG oxygens, and (C) the three membrane headgroup oxygens.

Table 2. Percentage of Cations in Each of the Four Available Environments

%	Na^+	K^+	Ca^{2+}
free ions	8.6	62.7	4.9
bonded to Cl^- anion	1.0	0.8	40.5
bonded with PEG oxygens (PEG-O)	66.4	25.0	0.0
bonded to lipid headgroup oxygens (H−O)	18.8	7.8	43.7
bridging between PEG-O and H−O	1.7	2.0	0.1
bridging between different PEG-O	3.6	1.6	0.0

weaker, indicating a larger fraction of the K^+ ions free in solution.

The number of bound cations to the same three electronegative sites, and cations free in solution, the four possible environments, was calculated using a formalism we had developed in previous work,[19] and these results are shown in Table 2. We see, in agreement with the previous observations and calculations, no Ca^{2+} bound to the PEG oxygens, a far larger number of Na^+ cations bound to PEG oxygens, and far fewer free Na^+ than K^+ ions. The Ca^{2+} ions have a much more dominant interaction with their anion partners than the Na^+ and K^+ cations. It must be stressed that for the case of the Ca^{2+} ions both the PEG layer forms a very large effective potential barrier, and the mobility of the individual Ca^{2+} ions between different environments is very low, and thus it is not possible to obtain sufficient statistics to obtain an equilibrium density of states for the Ca^{2+} ions; therefore, we can only say that both the membrane headgroups and outside the PEG region are both favorable environments for the Ca^{2+} ions, and cannot comment on the relative favorability of these two environments.

DISCUSSION

In a previous study,[11] we simulated PEGylated membranes in both the liquid crystalline and gel states at a molar concentration of PEGylated lipid in the membrane formulation of 1:9. In this previous study, we found (1) Na^+ ions to strongly associate with the PEG polymer and (2) for the case of the liquid crystalline, but not the gel, membrane the PEG polymer penetrated into the hydrophobic membrane interior. As a result of both item 2 and the increased area per headgroup of the liquid crystalline membrane, the PEG layer was considerably less dense for the liquid crystalline membrane than for the gel membrane. The Cl^- ions were found to collocate to the PEG layer for the case of the liquid crystalline membrane, where the reduced density allowed the Cl^- ions to be surrounded by

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water shells. For the more dense PEG layer of the gel membrane The Cl^- ions were excluded from the PEG layer, and an effective double charge layer resulted. The current study builds on this insight by studying the effect of altering the PEG and salt concentration, and the type of salt present.

We studied the effect of salt concentration by simulating the PEGylated membrane with only Na^+ counterions (one for every PEGylated lipid) as opposed to the physiological salt concentration. In absence of salt, the Na^+ cations, while still binding to the PEG and membrane headgroups, present a different profile. The Na^+ ions bound by the PEG are located deeper in the PEG region (closer to the membrane headgroups) for both the liquid crystalline and gel membrane systems without salt. This is exactly as one would expect from basic theory regarding the effect of addition of an electrolyte to the solution (Poisson−Boltzmann equation) between two parallel charged plates in the presence of counterions.[31] We also see that for both the liquid crystalline and gel systems the thickness of the PEG layer is expanded slightly by the presence of salt. This can be explained by the greater number of cations bound, increasing the electrostatic repulsion within the layer. This result is in agreement with a study of Vukovic et al.[32] where they found both experimental evidence and results from an atomistic simulation that demonstrate an inflation of the PEG corona of the micelle with increasing salt concentration. For the liquid crystalline system we observe that the extent to which the PEG polymer penetrates into the membrane interior is also increased by the presence of salt.

Regarding the effect of altering the salt from NaCl to either KCl or $CaCl_2$ we see a clear qualitative change in the interaction between salt ions and the PEGylated membrane surface. The PEG interaction with the K^+ ions was weaker, a lower portion of K^+ ions was within the PEG region relative to the case for Na^+ and, unlike the case for Na^+ ions, a substantial portion of the K^+ ions remained free in the solution, and as a result the strength of the electrostatic potential barrier peaks at both the PEG region and the membrane headgroup peaks was decreased for the KCl system with respect to the NaCl system. That the interaction with PEG should differ between different monovalent ions is not without precedent. Crown ethers are chemically identical to PEG, only formed in rings of fixed diameter. It is well-known that setting the diameter of the crown ether alters the selectivity for different cations;[33] in fact, Charles Pederson shared the 1987 Nobel prize in chemistry for finding this. The PEG chain has a natural curvature that will fit more comfortably around one ion than another, and it is known to interact more strongly with Na^+ than K^+ ions.[34] This results from the differing effective sizes, and van der Waals interaction strengths, of the ions. We also see an effect on the charge double layer strength with changing ion type that will be roughly proportional to the height of the electrostatic potential peak in the region of the PEG layer. The charge double layer can be seen to be significantly stronger for the PEGylated bilayer in the NaCl solution than either the KCl or $CaCl_2$ solutions.

Regarding the system with $CaCl_2$ as salt, the result was yet more striking: while the Ca^{2+} ions associated strongly with the membrane headgroups, they did not collocate to the PEG region or associate with the PEG oxygens in any way. While initially surprising, this result is in fact supported by experimental evidence in the literature. Bronich et al.[35] used Ca^{2+} to cross-link the hydrophobic cores of PEGylated micelles. They saw no cross-linking between separate micelles due to the Ca^{2+} ions. The clearest evidence is found by Sondjaja et al.,[36] where they found that the percentage of free Ca^{2+} in solution varies linearly right down to zero concentration in the presence of PEO homopolymer. In addition, Holland et al.[37] found evidence that Ca^{2+}-induced liposome fusion is inhibited by PEGylation. The mechanism for Ca^{2+}-induced fusion is cross-linking between lipid headgroups which are known to strongly bind Ca^{2+} ions.[18,38,39] Our result shows a mechanism for the experimental results found by Holland et al.: the Ca^{2+} ions will still bind to the lipid headgroups; however, the PEG layers, which do not associate with the Ca^{2+} ions, will act as a steric barrier to fusion.

At this point, it is appropriate to summarize what is occurring with the three cation varieties. There are essentially four competing environments for the ions: the headgroup oxygens, the PEG oxygens, the water oxygens, and the Cl^- counterions. Apart from the charge of the ions, they also have a characteristic size that either favors or inhibits their presence in certain surroundings. A combination of entropic and enthalpic processes drives one or another of these four environments to be more or less favorable to the presence of these ions. This difference in the free energy in each of the environments is dependent on the effective size and van der Waals interaction strength of the different ion types.

We found that a reduction in the formulation density from 1:9 to 1:18 in the gel membrane has the same effect as a transition from gel to liquid crystalline phase at the formulation density of 1:9: the less dense PEG layer accommodates the Cl^- ions in water pockets, and thus the extent to which the Cl^- ions are able to penetrate the membrane is increased. The Cl^- ions interact strongly with water and, as a result, have an effectively bound shell of water molecules surrounding them. Since separating the hydration shell from the Cl^- ion involves too high an interaction penalty, sufficiently large spaces within the PEG layer must exist for the Cl^- ions with their hydration shell to fit. An increase of the extent to which the Cl^- ions penetrate the membrane will in turn result in greater overlap of the mass density peaks of the Cl^- ions with the Na^+ ions in the PEG layer, and thus a decrease of the charge double layer strength. Our results for the electrostatic potential along the membrane normal bear this out: the electrostatic potential peak at the PEG layer is significantly stronger for the 1:9 than for the 1:18 system.

As we noted in our previous publication, this change in the nature of the PEG layer with increasing density, resulting in an increase in the charge double layer at the liposome surface, may be of pharmaceutical significance: it is known that surface charge plays a role in liposome uptake by the RES, a charged surface of the liposome enhancing opsonization, thus possibly increasing the elimination rate.[5] The exact mechanism through which PEGylation inhibits RES uptake of liposomes is not clearly understood. The first step of complement activation by the RES is opsonization,[5] where a set of bloodstream proteins adhere to the outside of particles and identify them so they can be eliminated by macrophages, and one school of thought is that PEG inhibits this. Our result indicates that, up to a certain PEG density, the surface of the liposome, positively charged without PEG, is protected by an effectively neutral polymer layer. Beyond a certain density, however, the PEG layer becomes effectively charged. It is possible that this mechanism may in part explain the conflicting experimental results found in the literature regarding the effect of PEGylation on liposome−protein interactions.[7,9,10,40−46] On the other hand, while some

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studies indicate that PEGylation inhibits protein adhesion,[9] other studies have found this not to be the case.[10] Alternative mechanisms that have been proposed include inhibition of liposome fusion[46] and acting as a direct steric barrier against macrophages.[10] If the mechanism is in fact inhibition of liposome fusion, then we may have an explanation as to why PEG has been found to work so particularly well as a protective polymer coating: the lack of interaction between PEG and Ca^{2+} that is due to the particular structure of PEG results in inhibition of Ca^{2+}-induced liposome fusion. Whether or not calcium-induced fusion is the dominant mechanism for rapid clearance of liposomes from the blood plasma, the effect of PEGylation on the liposome properties that our results demonstrate is of significance: for example, since cell culture media contain Ca^{2+} ions, these fusion-related events are relevant for in vitro cell experiments.

CONCLUSION

In building on our previous work,[11] we have used all-atom molecular dynamics simulations of PEGylated membranes to obtain a comprehensive model of the structure and interaction with salts found in physiological conditions of the PEGylated liposome surface. We found that addition of salt slightly expands the PEG layer and expands the region of the PEG layer where the Na^+ ions are located, in qualitative agreement with Poisson−Boltzmann theory regarding charge distribution near a charged plane in the presence of an electrolyte. For the liquid crystalline membrane, the PEG polymer also penetrates deeper into the membrane when salt is added. When the molar density of PEGylated lipids in the formulation is reduced from 1:9 to 1:18, we see the same effect as altering the membrane phase from gel to liquid crystalline at 1:9 molar density: the Cl^- ions, excluded from the PEG region at 1:9 molar density, are able to collocate to the PEG surrounded by water pockets in the PEG layer large enough to incorporate them. When the NaCl was replaced by KCl or $CaCl_2$, the interaction between PEG and cations was altered qualitatively: the interaction between K^+ ions and PEG was weaker than the case for Na^+ ions, and the majority of K^+ ions were found to be free in solution. The Ca^{2+} ions were found to interact very strongly with the membrane headgroups and Cl^- anions but exhibit no binding to PEG, thus presenting a mechanism for the experimentally observed[37] role of PEGylation in inhibiting calcium-induced liposome fusion.

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Notes

The authors declare no competing financial interest.

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