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Key Factors Regulating the Mass Delivery of Macromolecules to Model Cell Membranes: Gravity and Electrostatics

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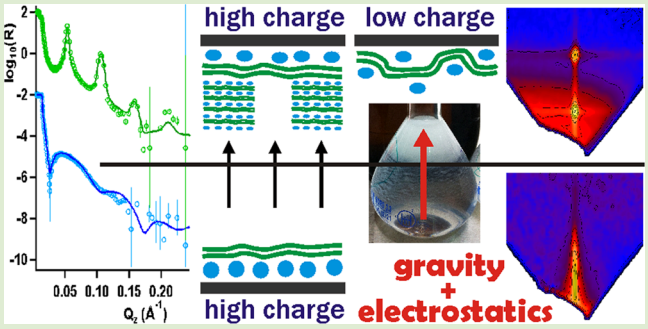
Supporting Information

ABSTRACT: We show that both gravity and electrostatics are key factors regulating interactions between model cell membranes and self-assembled liquid crystalline aggregates of dendrimers and phospholipids. The system is a proxy for the trafficking of reservoirs of therapeutic drugs to cell membranes for slow diffusion and continuous delivery. Neutron reflectometry measurements were carried out on supported lipid bilayers of varying charge and on hydrophilic silica surfaces. Translocation of the macromolecule across the membrane and adsorption of the lamellar aggregates occur only when the membrane (1) is located above the bulk liquid and (2) has sufficient negative charge. The impact of such dramatic directionality effects due to bulk phase separation and gravity is emphasized for future biochemical investigations. Further, the potential to switch on the interaction mechanism through tuning the charge of the aggregates to activate endocytosis pathways on specific cell types is discussed in the context of targeted drug delivery applications.



Scientist worldwide aim at optimizing the interaction of therapeutic drugs with cellular membranes, and in this Letter we outline how gravity and electrostatics can be key parameters for the focus of future research.

An immense effort has been invested to understand the mechanisms of interactions between macromolecules and cell membranes.[1,2] Positively charged macromolecules can be recruited into the cell by the endocytosis pathway[3] and then trafficked by different organelles according to their surface charge.[4] A key challenge is to develop drug delivery systems involving the efficient transport of therapeutic agents to lipid membranes.[5] It can be advantageous to position reservoirs of the drug in contact with the membrane for continuous delivery by slow diffusion.[6] In this case the drug may be encapsulated into aggregates of liquid crystalline phase,[7] which is a technique already exploited in clinical studies.[8]

Membrane interactions between macromolecules and lipid vesicles as model cell membranes have been studied by isothermal titration calorimetry,[9] nuclear magnetic resonance,[10] and dynamic light scattering,[9,11] among others. Supported lipid bilayers (SLBs) on solid substrates are also used to study such interactions using methods such as neutron scattering,[12,13] X-ray scattering,[14] ellipsometry,[12,14] atomic force microscopy,[9−11,15] spectroscopic techniques,[16,17] and molecular dynamics simulations,[9,18] among others. Possible mechanisms include the target adsorbing to the SLB,[12,13] translocating across the SLB,[12] forming interfacial multilayers,[13] or even destroying the SLB.[9,10]

The present work concerns interactions with model membranes of lamellar aggregates comprising phospholipids and macromolecules with strong biomedical potential. The aim of the work is to highlight key factors that regulate the efficient delivery to SLBs of large reservoirs of macromolecules. Factors under consideration are (1) the location of the interface with respect to the gravitational field and (2) the electrostatic interactions between the aggregates and the SLB.

The system chosen for study is lipid vesicles comprising a mixture of 90 mol % 1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC; zwitterionic) and 10 mol % 1-palmitoyl-2-oleoyl-phosphatidyl-glycerol (POPG; anionic) combined with poly-(amidoamide) (PAMAM; cationic) dendrimers of generation 6. A lipid-to-dendrimer molar ratio of 130 was used. While there is a delicate balance between capability and toxicity of drug carriers,[19] dendrimers are good candidates as drug delivery vectors due to their ability to translocate across cellular membranes.[11,12,15] The mixture chosen separates into a lamellar liquid crystalline phase and excess vesicles in the bulk.[20] Lyotropic phases are currently being studied extensively due to

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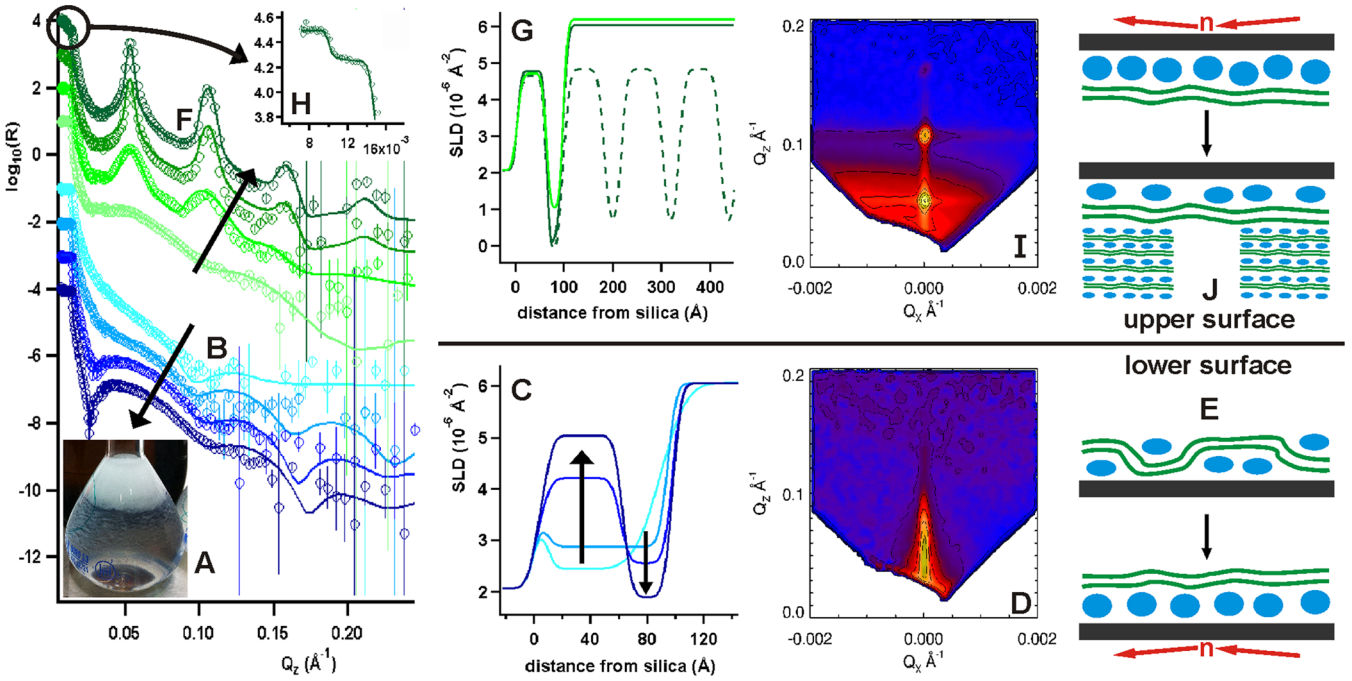

Figure 1. (A) POPC/POPG/PAMAM sample after 9 h showing phase separation; NR data and fits of hydrophilic silica surfaces (B) below and (F) above the POPC/POPG/PAMAM sample with respect to the sample age (2, 5, 16, and 30 h with increasing darkness), where data sets and fits are offset in reflectivity for clarity, and their respective scattering length density profiles for silica surfaces (C) below the sample after 2, 5, 16, and 30 h and (G) above the sample after 2 and 30 h where the full and dashed dark lines are contributions to the reflectivity from the two domains described in the text; (H) magnification of the data and fit involving an incoherent addition model for the double critical edge of the 30 h data in F; off-specular scattering plots of $Q_z(Q_x)$ with a logarithmic intensity scale for surfaces (D) below and (I) above the sample after 30 h; schematics indicating the change in interfacial structure from 2 to 30 h for surfaces (E) below and (J) above the sample where black slabs are silicon crystals, blue ovals are dendrimers, green double lines are bilayers, the edge of each crystal facing the adsorbed molecules marks "zero distance" in C and G, and red arrows depict the reflection of neutrons. Drawings are not to scale. The gap between the two bound lamellar aggregates in J is solvent. Repeating structure in G continues for 100 repeating units. Straight black arrows mark the passing of time.

their potential to encapsulate large quantities of molecules relevant for delivery over long exposure times.[21]

The mechanism of membrane interaction is of key importance with reference to any potential delivery application. For the dendrimer size and lipid-to-dendrimer molar ratio used in the present study, Kelly et al. predicts the adsorption of flattened dendrimer molecules to the SLB rather than lipid extraction or micelle formation.[9] Equally, Smith and co-workers showed that dendrimers are thermodynamically stable in the core of zwitterionic lipid bilayers even though strong kinetic barriers were proposed.[10] A dendrimer translocation mechanism across the lipid bilayer was prevalent in studies involving SLBs with negative charge by Ainalem et al.,[12] even though Åkesson et al. observed only adsorption.[13]

The primary experimental technique used is neutron reflectometry (NR), which is sensitive to the adsorbed layer structure (isotopic substitution), the presence of interfacial multilayers (Bragg diffraction peaks), and in-plane surface arrangements (off-specular scattering). We used an approach involving the reflection up versus down modes of FIGARO at the Institut Laue-Langevin (ILL, Grenoble, France) with surfaces located above and below the liquid.[22] The reason for this approach is that different properties were previously revealed on surfaces located above and below a synthetic polyelectrolyte/surfactant mixture due to the transport under gravity of bulk aggregates,[23] and we sought to probe the significance of such effects to the present system of biomedical relevance. Phase separation occurs with normal hydrogenated liquid due to the lower density of the aggregates, but we used D_2O both to enhance the scattering contrast and to hasten phase separation. The layer structure was resolvable thanks to distinctive features in the specular reflectivity profiles. The sample was exposed to hydrophilic silica surfaces and to SLBs formed by vesicle fusion of varying ratios of the same lipid mixture; further details of the experimental methods may be found in part 1 of the Supporting Information, and additional NR details may be found in part 2.

We start our results with a study of the interactions of a fresh POPC/POPG/PAMAM sample with hydrophilic silica surfaces located above and below the bulk liquid. Immediately upon mixing, the sample is turbid due to the presence of micrometer-sized bulk lamellar aggregates. The aggregates are slightly positive with a conductivity of 0.19 ± 0.04 μS/cm; further details of these measurements may be found in part 3 of the Supporting Information. The suspension is not stable as spatial separation of a condensed phase above a dilute phase occurs with time due to gravity (Figure 1A).

The dilute phase of the lipid/dendrimer mixture interacts with the lower surface. After 2 h, the interfacial structure was approximately 90 Å thick, but the dendrimers and the lipids could not be discerned into defined layers, probably due to the coexistence of domains of dendrimer-below-bilayer and bilayer-below-dendrimer. With time, dendrimer molecules bound to the outer leaflet of the SLB slowly translocated across the membrane. After 30 h, a dendrimer layer (thickness 54 Å; coverage 29%) was in direct contact with the silica and a lipid bilayer (thickness 35 Å; coverage 65%) was floating on top of it (Figure 1B,C). This structure is in keeping with the flattened

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dendrimer model described by Kelly et al.[9] and Åkesson et al.,[13] and the changes are similar to the translocation observed by Ainalem et al.[12] The lack of Bragg peaks in the specular reflectivity data (Figure 1B) and the absence of off-specular scattering, otherwise exhibited as horizontal lines in a $Q_z(Q_x)$ projection of the neutron data (Figure 1D), suggests that (1) bulk lamellar aggregates did not adsorb to the surface, and (2) the surface itself did not template a multilayer self-assembly. A schematic of the interfacial structure at the lower surface is shown (Figure 1E).

The corresponding interaction of the condensed phase with the upper surface is dramatically different. Four Bragg diffraction peaks in the specular NR data reveal that a lamellar stack of lipid/dendrimer repeating layers is in contact with the surface (Figure 1F,G). Further, a close inspection of the data at low vertical momentum transfer values (Q_z) shows that there are two contributions to the total reflection resulting in a double critical edge (Figure 1H). Fitting the two critical edges in the data required a model involving the incoherent addition of contributions to the reflectivity from two macroscopic domains of different surface structures. The length scale for the domains is above the neutron coherence length of the measurement (~10 μm); further details of the model applied may be found in part 4 of the Supporting Information.

After 30 h, the structure of the first domain, with 55% area coverage, is similar to that on the lower surface with a dendrimer layer (thickness 55 Å; coverage 40%) and a floating lipid bilayer (thickness 35 Å; coverage 80%). The second domain, with 45% area coverage, involves macroscopic stacks of dendrimers (thickness 84 Å; coverage 35%) with lipid bilayers (thickness 35 Å; coverage 90%). This latter structure matches the lamellar spacing for the bulk liquid crystalline aggregates measured by small-angle X-ray scattering.[20] The number of layers in contact with the membrane cannot be resolved due to limitations of the instrument resolution, but the structure is at least hundreds of nanometers thick and is consistent with the micrometer length scale of the bulk aggregates.[20] Interestingly, even after just 2 h when there are no Bragg peaks or split critical edge in the data, the near-surface structure is very similar to that of the first domain with a dendrimer layer (thickness 55 Å; coverage 36%) and a floating lipid bilayer (thickness 33 Å; coverage 94%). Hence, even at the very early stages of the interaction, the interfacial properties depend on the location of the surface with respect to the gravitational field. The physical picture for the interfacial arrangement is that of intact micrometer-sized aggregates from the bulk which do not rearrange and fuse to form a homogeneous surface lamellar phase.

It is unclear from the specular NR data alone whether the process involves (i) adsorption of bulk aggregates driven by surface affinity or (ii) disordered and nonspecific accumulation of bulk aggregates driven by buoyancy. Information about the degree of alignment of the aggregates can be obtained from off-specular neutron scattering, which results from in-plane correlations along the interface. In spite of the strong Bragg peaks in the specular reflection (bright dots on the vertical Q_x = 0 axis), the off-specular scattering (horizontal lines emanating from the Bragg peaks) with respect to the specular reflection is 40 times weaker than data recorded for a disordered system[23] (Figure 1I); further details about the off-specular scattering comparison may be found in part 5 of the Supporting Information. We may infer therefore that while transport of the aggregates to the near-surface region occurs due to phase

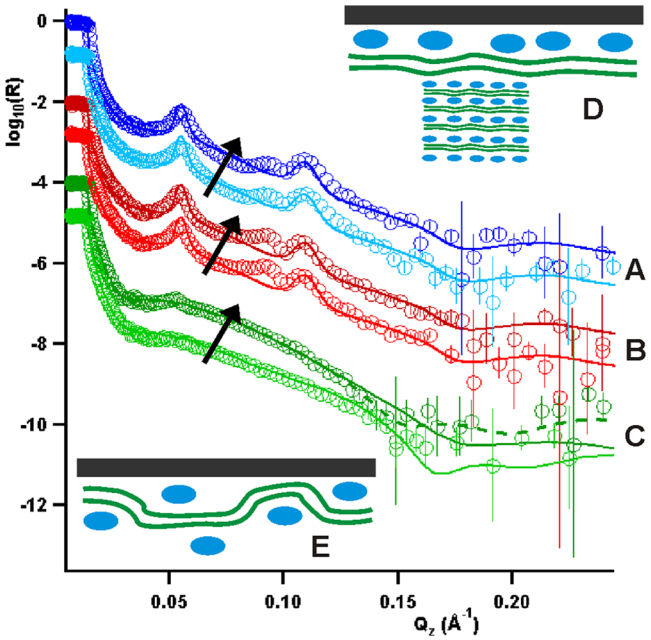

Figure 2. Specular NR data and fits of the interaction of POPC/POPG/PAMAM mixtures with preformed SLBs from vesicles with (A) 25, (B) 17.5, and (C) 10 mol % POPG in POPC after (light color) 12 h and (dark color) 30 h. Data sets and fits are offset in reflectivity for clarity. The small Bragg peak at 0.090 Å^{-1} result from residual multilamellar vesicles in the sample used for experiments A and B. For the fit to (C) the filled line is a 2-layer dendrimer/lipid model and the dashed line is a 3-layer dendrimer/lipid/dendrimer model. Schematics of the layer structure for the (D) 17.5/25 mol % experiments and (E) 10 mol % experiment are shown. Drawings are not to scale. Scattering length density profiles corresponding to the 30 h data may be found in part 2 of the Supporting Information.

separation and gravity, the surface interaction is driven by adsorption, that is, surface affinity. A schematic of the interfacial structure at the upper surface is shown (Figure 1J).

We turn our attention now to the interactions of the bulk lipid/dendrimer aggregates with preformed SLBs made from the fusion of vesicles comprising 10, 17.5, and 25 mol % POPG in POPC (Figure 2). As the bulk aggregates had been shown to float, these measurements were carried out only on SLBs located above the bulk liquid. The preformed SLBs all had excellent coverage of >95%.

Bragg peaks are present in the specular NR data for SLBs made from 25 and 17.5 mol % charged lipids (Figure 2A and B, respectively) and the data are consistent with the coexistence of two different surface structures on the micrometer scale. The structure involved domains of 98% area coverage with a translocated dendrimer layer (thickness 45 Å; coverage 70%) and a floating lipid bilayer (thickness 35 Å; coverage 90%), and domains of 2% area coverage with adsorbed lamellar aggregates (structure as above). The kinetics of the interaction were fast: the dendrimer layer was fully translocated within 30 min, and the bulk aggregate adsorption process had reached steady state after just 2 h. Also, the high coverage of dendrimer is consistent with the greater amount of negatively charged lipid in the bilayer and the higher ionic strength compared to the results of Ainalem et al.[12] The Bragg peak intensities and rate of growth are similar in the cases of the SLBs formed from 17.5 and 25 mol % negatively charged lipids. This may be explained by near-neutrality of the outer leaflet of the floating bilayer exposed to

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the aggregates in both cases after the negatively charged lipids selectively locate in the inner leaflet adjacent to the translocated, positively charged dendrimer molecules. The final interfacial structure determined is shown schematically in Figure 2D.

For the SLB made from 10 mol % POPG (Figure 2C), the lamellar stacks are practically absent, and only a small first-order Bragg peak at Q_z = 0.055 Å^{-1} is observed after 30 h. The surface structure fits reasonably well to a translocated dendrimer layer with a floating lipid bilayer (filled line), but the fit improved slightly when including a sparse additional dendrimer layer on top of the bilayer (dashed line). Even after 30 h, the surface excess of dendrimer in contact with the silica is only about half that of the higher charged SLBs. This indicates that the translocation kinetics are suppressed by the low content of negatively charged lipid. At earlier times, the surface structure comprised an undefined layer of dendrimer and lipid with a total thickness of 90 Å, consistent with the coexistence of domains of dendrimer-below-bilayer and bilayer-below-dendrimer. A physical picture for the interaction is initial adsorption of dendrimer molecules to the SLB followed by a slow translocation process taking many hours. While the translocation process is incomplete, a net positive charge remains exposed to solution from dendrimers bound to the SLB and there is an electrostatic barrier to adsorption of the bulk lamellar aggregates. As the translocation process becomes advanced, the net positive charge is reduced and the adsorption of the bulk aggregates begins. The layer structure is shown schematically in Figure 2E.

Smith et al. showed that intercalated dendrimer molecules are thermodynamically stable in zwitterionic lipid bilayers when premixed in nonaqueous solvent and observed no interaction with the headgroups.[10] In the present work, we tracked kinetically the slow interaction of the dilute phase of the PAMAM/POPC/POPG sample with silica. A structure primarily consisting of surface lipid bilayer + adsorbed dendrimers was replaced by regions of surface dendrimers + floating lipid bilayer. Throughout the process, the total layer thickness remained 90 Å (even though the lipid coverage decreased slightly), which corresponds to one bilayer and one flattened dendrimer layer as predicted by Kelly et al.[9] At no point was there a change in interfacial layer thickness that would indicate directly dendrimer molecules stably intercalated in the core of a single bilayer. In spite of this result, domains of dendrimers and bilayer are clearly located in the same plane, either localized in pores at the side of a distorted lipid bilayer or in contact with the core of bilayer as described by Smith et al.[10] The NR technique does not have the lateral resolution to distinguish the two scenarios. Further, it remains a possibility that the intercalated structure is an intermediate state of key importance in the translocation mechanism for membrane interactions of dendrimers.

It is also interesting to consider the completely different surface structures formed after the same lipid/dendrimer mixture had been in contact with the bare silica surface (Figure 1F) and the preformed SLB formed from lipid vesicles of the same composition (Figure 2C). The fact that reservoirs of macromolecules were in contact with the membrane only in the former case, as shown by the split critical edge and prominent Bragg diffraction peaks, highlights the nonequilibrium nature of the interaction involving the preformed SLB of modest negative charge. The Gibbs energy is expected to be lowest when the cationic dendrimer molecules are bound to both the hydrophilic silica surface and the negatively charged SLB, as understood both by electrostatic arguments and the entropic gain for release of bound counterions. It follows therefore that the reduced lamellar surface structure observed is a consequence of the slow dendrimer translocation kinetics. Further, the fact that the macromolecule delivery mechanism is active with just a few percent more charged lipids in the SLB (Figure 2A,B) indicates the potential to tune this interaction in vivo.

To summarize our results, the exposure of the lipid/dendrimer mixture to the bare hydrophilic silica surface revealed that the cationic macromolecules bind rapidly, a floating lipid bilayer forms on top, and only in the case of the upper surface there is adsorption of bulk lamellar aggregates. Gravity and bulk phase separation determine the selectivity for adsorption of reservoirs of macromolecules to the upper surface. A similar surface structure results when the same particles interact with a preformed lipid bilayer as long as it carries sufficient negative charge. In this case the negative lipids accelerate the translocation of the macromolecules across the SLB, thus, reducing the positive charge exposed to the solution and allowing the aggregates to adsorb. Electrostatic interactions govern the rate of translocation, which is key for the attachment of the reservoirs of macromolecules.

Our findings have important implications for drug delivery investigations and applications. First, while there is routine research in the investigation of potential new drugs to interact with cell membranes, and formulations often involve complex mixtures, effects of gravity on membrane interactions are probably neglected in the vast majority of cases. The present study, where two interfaces of the same sample exhibit totally different behavior, emphasizes the importance of understanding such effects. The message is especially pertinent for researchers involved in studies where only microliters of a sample may be prepared and hence the implications of bulk phase separation on the interfacial properties may not be apparent. While we exploited the high density of D_2O to accelerate the delivery process, sucrose-based mixtures could also be used to tune the same physical effects. These findings may be relevant to consider in the context of floating drug delivery therapies that exploit buoyancy to increase gastric retention times in the stomach.[24]

Second, as negative surface charge on membranes has been shown to direct positively charged macromolecules into the endocytosis pathway,[3] there is scope to trigger this mechanism in drug delivery systems via attachment of lamellar aggregates to the cell. We have demonstrated using a lyotropic phase of lipids and cationic macromolecules that electrostatic interactions determine the rate of translocation of macromolecules across a model membrane, the process of which in turn triggers the mass adsorption of reservoirs of macromolecules from the bulk. It follows that fine adjustments of the proportion of charged lipids to macromolecules in a given drug delivery formulation can be carried out to optimize its performance. Further, given the different charge densities of healthy and cancerous cells in the body,[25] there is potential for tuning the electrostatic nature of therapeutic agents to gain selectivity in targeted deliveries to specific cell types. Such tuning of the electrostatics may be considered in relation to other factors such as the molecular weight of the macromolecule[15] and the fluidity of the targeted membrane.[26] These factors may be particularly relevant given that it was shown recently that small

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dendrimer molecules have potential as a delivery vector in biomedical treatments of the skin.[27]

This Letter has shown that, through gaining an understanding of the roles of gravity and electrostatics in a complex biomedical interaction, there is potential for the slow diffusion of macromolecules from lyotropic phases to cell membranes to be a future focus for drug delivery research.

■ ASSOCIATED CONTENT

Supporting Information

Further experimental details, additional NR details, conductivity results, NR incoherent addition model, and off-specular neutron scattering analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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