Ligand–receptor binding on nanoparticle-stabilized liposome surfaces

Liangfang Zhang,[a] Kevin Dammann,[b] Sung Chul Bae[b] and Steve Granick*[abc]

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We explore the access of receptor (streptavidin) to liposome-immobilized ligand (biotin) in cases where the liposomes are stabilized against fusion by allowing nanoparticles to adsorb. It is found that receptor binding persists over that range of nanoparticle surface coverage where liposome fusion and large-scale aggregation are prevented. This indicates that liposome outer surfaces, in the presence of stabilizers, remain biofunctionalizable, and may have bearing on explaining the long circulation time of stabilized liposomes as drug delivery vehicles.

Introduction

The design of function in phospholipid vesicles (also called liposomes) is complicated by contrary needs. On the one hand, their stability against fusion with one another is augmented by coating them with a protective layer (for example, PEG, poly(ethylene glycol)). On the other hand, the capability of vesicles to react chemically with their environment requires that reactants have access to the vesicle surface. Here, with direct experiments, we address this trade-off. How is protein-membrane binding modulated by repulsion that prevents deleterious aggregation or fusion of neighboring liposomes?

The context of this dilemma is that liposomes have been widely considered as drug delivery vehicles because of their ability to encapsulate many kinds of therapeutic agents with higher carrying capacity than can be reached using alternative drug carriers such as polymers, nanoparticles and hydrogels. However, it is difficult to minimize recognition and clearance of liposomes by phagocytic cells of the reticuloendothelial system; that is, to prolong the liposome circulation time.[1–4] Pioneering studies found that surface-grafted flexible and hydrophilic polymers such as polyethylene glycol (PEG) substantially lessen blood clearance of liposomes, the most plausible interpretation being that plasma protein adsorption to PEGylated liposomes is suppressed by the repulsive steric obstacles presented by the grafted polymer.[5–7] The generality of that interpretation is called into question by recent adsorption and clinical findings showing that even though PEGylated liposomes are macrophage-resistant, they are unlikely to impede protein adsorption.[8–10] To put into context these controversial findings, it is desirable to understand how protein–membrane binding is modulated by the repulsion that prevents deleterious aggregation or fusion of neighboring liposomes.

We investigate this question using a newly-designed stabilized liposome system—nanoparticle-stabilized phospholipid liposomes.[11] Earlier, we reported that the presence on the liposome surface of physisorbed, charged nanoparticles at submonolayer surface coverage appears to form "studs" (electrostatic and steric in origin) that armor the vesicles against fusion with one another[11] and with various substrate surfaces,[12] thereby producing liposomes the shelf life of which is at least several months, though they have not yet been tested *in vivo*. One potential advantage of this stabilization strategy is that the strength of the repulsion between liposomes is easily tuned by varying the surface coverage of adsorbed nanoparticles. The experiments described below explore the dependence on surface coverage. First, in the case that nanoparticles adsorb sparsely, we confirm earlier experiments of others showing that streptavidin proteins not only bind to liposomes containing embedded biotin ligands but also can bridge between liposomes to form liposome networks or clusters.[13] The new point is that in the case of higher levels of nanoparticle surface coverage, large-scale aggregation between liposomes fails to occur but ligand–receptor binding remains effective.

Experimental

For study, we selected the phospholipid DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine. Small unilamellar lipid vesicles (liposomes) were prepared using the well-known extrusion protocol.[14] They were prepared in PBS buffer (10 mM, pH = 6.0) to have a diameter of 200 nm. Biotinylated DPPE lipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl), was doped into the liposome phospholipid bilayer membrane in small abundance, typically ~1000 molecules per liposome on average, to form biotinylated liposomes. Both lipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Carboxyl-modified white polystyrene (PS) latex, the surface of which was hydrophilic and negatively charged, was purchased from Interfacial Dynamics Corp. (Eugene, OR).

Using methods described elsewhere,[11] liposomes of DLPC, diameter 200 nm, were prepared at 1 vol% concentration and mixed by low-power sonication with negatively charged PS nanoparticles, diameter 20 nm. The molar ratio of 100 : 1, approximately 1 : 1 by weight, corresponds to ~25% surface coverage if all nanoparticles adsorb. Streptavidin protein labelled with Rhodamine B was purchased from Molecular Probes Inc. (Eugene, OR).

Measurements of streptavidin diffusion were performed using fluorescence correlation spectroscopy (FCS) in the 2-photon excitation mode.[14] In a home-built apparatus, near-infrared

[a]*Department of Chemical & Biomolecular Engineering, University of Illinois, Urbana, IL, 61801, USA*
[b]*Department of Materials Science and Engineering, University of Illinois, Urbana, IL, 61801, USA*
[c]*Departments of Chemistry, and of Physics, University of Illinois, Urbana, IL, 61801, USA. E-mail: sgranick@uiuc.edu*

femtosecond pulses from a Ti : Sapphire laser (800 nm, 82 MHz, pulse width ~100 fs) were focused onto the sample through a water immersion objective lens (Zeiss Axiovert 135 TV, 63×, N_A = 1.2), giving an excitation spot the diffraction-limited diameter of which was ~0.35 μm. Fluorescence was collected by the same lens and detected by a single photon-counting module (Hamamatsu). Fluorescence intensity–intensity autocorrelation curves were analyzed in traditional ways[15,16] to give the translational diffusion coefficient (D).

For imaging, epifluorescence microscopy was employed. Light (532 nm) from a diode-pumped Nd : YAG laser (CrystaLaser) was directed into an inverted microscope (Zeiss Axiovert 200) in an epi-illumination geometry. Fluorescence emission was collected through the same objective and detected by a back-illuminated electron multiplying charge-coupled device (CCD) camera (Andor iXon DV-887 BI) after removing the laser scattering by a 532 nm notch filter. The total system magnification of the CCD was 150 nm per pixel.

Results and discussion

First, we compare the translational diffusion coefficient (D) of streptavidin diffusing freely in aqueous solution with that of streptavidin mixed with biotinylated liposomes. Each liposome carried 100 nanoparticles on average. This corresponds, bearing in mind the sizes of the liposome (200 nm diameter) and nanoparticle (20 nm diameter), to ~25% surface coverage. The average streptavidin–biotin complex size is less than 5 nm, therefore, it is sterically possible to form the complex on liposome surfaces when they are sparsely covered by nanoparticles. As shown in Fig. 1, D of free streptavidin was 130 ± 10 $\mu m^2\ s^{-1}$. However, when the

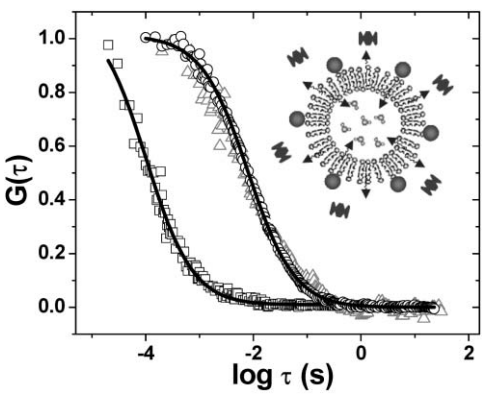

Fig. 1 Fluorescence intensity–intensity autocorrelation curve is plotted against logarithmic time lag τ when streptavidin labelled with Rhodamine B diffuses freely in aqueous solution (squares; data imply D = 130 ± 10 $\mu m^2\ s^{-1}$) or binds to nanoparticle-coated single unilamellar liposomes and diffuses while bound to these liposomes (circles; data imply D = 0.9 ± 0.2 $\mu m^2\ s^{-1}$). The autocorrelation curve in the latter case is indistinguishable from that for these same nanoparticle-stabilized liposomes in the absence of streptavidin (triangles). The stoichiometry of streptavidin to biotin in this example was 1 : 1 as described in the text and nanoparticle concentration in the system was such that if all nanoparticles adsorbed, each liposome would hold ~100 nanoparticles, amounting to an occluded surface area of ~25%. Inset: schematic illustration of single unilamellar vesicles containing dilute biotinylated lipids (black arrows), stabilized by adsorbed nanoparticles,[11] exposed to streptavidin in solution. Streptavidin comprises the only fluorescent element in this system.

streptavidin was mixed with the biotinylated liposomes at a 1 : 1 molar ratio to the biotin dwelling in liposome outer surfaces, D slowed by two orders of magnitude and was indistinguishable from D for these same nanoparticle-stabilized liposomes in the absence of streptavidin, showing that streptavidin bound to the liposome. A control experiment showed that streptavidin did not physisorb to the nanoparticles (see below). Therefore, streptavidin bound specifically to biotin located in the phospholipid membrane of the liposomes.

Next, bearing in mind that streptavidin possesses four active sites to which biotin can bind, we hypothesized that if the abundance of streptavidin was low, streptavidin might bridge between biotin groups on two different liposomes, linking them together. This is known to occur in the absence of nanoparticles[13] and was confirmed as a control experiment. As shown in Fig. 2A, in the absence of nanoparticles the liposomes aggregated to form large networks, large enough to settle from the solution in a few minutes. Since all fluorescently-labelled streptavidins bound to liposomes and settled to the surface, FCS measurements in bulk solution failed to give an autocorrelation curve. (Fig. 2 A).

Fig. 2 summarizes experiments in which the molar ratio of streptavidin to biotin staying in liposome outer surfaces was fixed at 1 : 5 and the abundance of nanoparticles was varied. First, for nanoparticle surface coverages of ~6% and ~12%, small clusters or other aggregates consisting of a few liposome units were formed (Fig. 2, panels B and C). The autocorrelation measurements reflected this polydispersity. Note that the FCS method is sensitive to liposome bridging because the liposome diameter is close to that of the FCS excitation spot. In the case of severe liposome aggregation, the resulting large clusters and polydisperse size distribution shifted and disturbed the FCS autocorrelation function in ways shown in Fig. 2, panels B and C.

Beginning at a surface coverage of ~25%, epifluorescence images revealed just discrete, individual liposomes, yet streptavidin bound to them without bridging between the liposomes. The translational diffusion coefficient was characteristic of the motion of single liposomes (Fig. 2D). At still higher surface coverage (~50%) streptavidin was not observed to bind; it stayed free in solution and formed a uniform fluorescent background (Fig. 2E). Parenthetically, this result also shows that streptavidin did not bind to nanoparticles. If this had occurred, individual liposomes would have been visible, as in Fig. 2D.

These results point to a possible interpretation of the macrophage-resistant property of stabilized liposomes,[8–10] even though the system described here differs structurally from PEGylated liposomes. Speculatively, we suppose that plasma protein may indeed adsorb in the presence of a steric substance, such as PEG,[5–10] or a charged substance, such as nanoparticles,[11] when their surface coverage is less than full. However, the binding of plasma proteins to these carriers does not dramatically increase their size, which (if it were to occur) would result in the carrier being rapidly recognized as a foreign body by phagocytic cells. Similarly, Discher and coworkers also found that the circulation time of PEG-coated polymersomes depends strongly on the coating properties of the PEG layer.[17]

This work also shows that at low surface coverage (<50%) the outer surfaces of nanoparticle-stabilized liposomes remain bio-functionalizable. This opens another door to using liposomes in emerging biotechnological fields in which it is desirable to possess

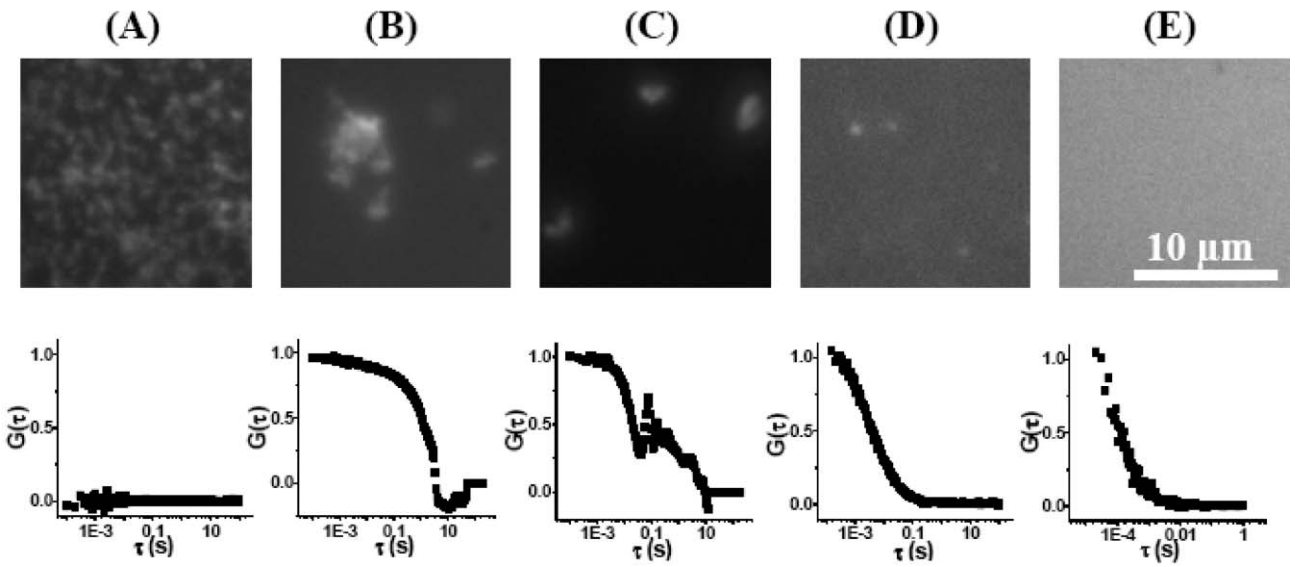

Fig. 2 Epifluorescence images (upper row) and fluorescence correlation spectroscopy (FCS) autocorrelation functions (lower row) showing how nanoparticle surface coverage on biotinylated liposomes influences streptavidin binding to them. The intensity–intensity fluorescence autocorrelation function of single measurements, normalized to its level at small time lag, is plotted against logarithmic time lag. Fluorescently-labelled streptavidin was mixed with phospholipid liposomes at the molar ratio of 100 to 1. On average, each liposome carries 500 biotin ligands on its outer surface, where a fraction of δ is covered by nanoparticles. (A) $\delta = 0$. Streptavidin bridges liposomes to form large aggregates that settle to substrate surfaces leaving no FCS signal in the bulk; (B) $\delta \approx 6\%$. Streptavidin bridges liposomes to form small clusters that enormously shift the autocorrelation function to longer times; (C) $\delta \approx 12\%$. Streptavidins bind to biotinylated liposomes and occasionally bridge them to form liposome dimers, trimers, tetramers and other aggregates consisting of just a few liposome units. This polydispersity highly disturbs the FCS autocorrelation function causing it to deviate from that characteristic of single-component Brownian motion; (D) $\delta \approx 25\%$. Streptavidin binds to biotinylated liposomes but no bridging is observed. Diffusion is the same as for discrete liposomes; (E) $\delta \approx 50\%$. Streptavidin fails to bind to biotinylated liposomes; it remains free in solution, forming a uniformly fluorescent background, and the FCS autocorrelation function is that of unbound streptavidin, which is about two orders of magnitude faster than that of nanoparticle-stabilized liposomes.

stable liposome suspensions while retaining the capacity for proteins embedded within the liposome to sense their environment.

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