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C2AB: A Molecular Glue for Lipid Vesicles with a Negatively Charged Surface

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Artificial particulate systems such as lipid vesicles are found in a variety of biomedical applications such as drug delivery and targeting. More versatile layers of control would be added if liposomes could be glued together on demand while stabilized against fusion. Here, we present a two-component molecular glue composed of a protein and calcium ions, with each component specialized for fast and specific binding to negatively charged lipid membranes. Upon mixing the two components, the high affinity binding of this glue starts to tightly bridge two lipid vesicles on a subsecond scale. Furthermore, highly charged liposomes are beneficial in preventing spontaneous fusion before applying the molecular glue.

Lipid vesicles (liposomes) have a flexible, cell-surface-like lipid bilayer surface which acts as a permeability barrier such that compounds can be entrapped in their aqueous interior. The surface of lipid vesicles is biofunctionalizable; that is, it can be tuned for attaching antibodies, protein receptors, and DNA molecules.[1,2] The liposome has also served as an excellent model membrane system for studying membrane proteins.[3−5] Furthermore, the compartments of lipid vesicles can be used to encapsulate and store various cargoes, such as enzymes, proteins, DNA, and various drug molecules.[6,7] Signal transduction embedded in liposomes or the performance of liposomal applications would be amplified if the liposomes could be clustered together upon need. One good example is the liposomal drug. We envision liposomes be stabilized against fusion before they arrive at the target region such as tumors. Such stabilization can be achieved by using primarily polyethylene glycol (PEG) molecules or via charged lipids, DNA molecules, or nanoparticles.[8,9] After the liposomal drugs accumulate around the target cells, clustering of liposomes to create a high local concentration would be pivotal for therapeutic efficacy.

Here, we report a molecular glue for fast (< 1 s) and specific clustering of lipid vesicles. The working principle of this glue is similar to that of the epoxy glue, which involves one agent named C2AB and calcium ions as the second agent. The fast and strong adhesion can be achieved by applying them to the charged liposomes. In this paper, a high concentration of negatively charged lipid molecules is utilized for increasing the adhesiveness of the glue; meanwhile, it produces a strong repelling force to prevent spontaneous fusion in the stock solution. Based on our experience, liposomes contain 35 mol % negatively charged phosphatidylserine (PS) molecules and 65 mol % neutral phosphocholine (PC) can be stable at least 2 weeks at the concentration of ∼100 nM.

The molecular glue, C2AB, is the cytosolic part of synaptotagmin I, believed to be the primary calcium sensor in synaptic vesicle exocytosis, and has two C2 domains referred as the C2A and C2B domains.[10,11] The C2A domain binds three calcium ions, whereas the C2B domain binds two calcium ions with their calcium binding pockets formed by two protruding loops at the top of the beta sandwiches (Figure S1 in the Supporting Information). The C2AB molecule interacts with both SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes, which is an essential part of the intracellular membrane fusion machinery, and negatively charged phospholipids, such as phosphatidylserine (PS). In response to the calcium, C2AB partially penetrates the membrane to deform the lipid bilayer and lower the activation barrier to assist the membrane fusion.[12,13] The detailed mechanism of C2AB binding on membranes with assistance from calcium ions has been studied by Rizo's group.[13] Simply, after calcium ion binding, C2AB molecules containing multiple binding sites become very positively charged, which has a high affinity with negatively charged lipid molecules.

Lipid vesicles with a diameter of 100 nm were produced according to the standard extrusion protocol with materials obtained from Avanti Polar Lipid, Inc. (Alabaster, AL). As we stated above, to enhance the affinity of C2AB molecules to lipid membranes, liposomes were made of a relatively high concentration of PS lipids, 35 mol %, plus 65 mol % phosphocholine,

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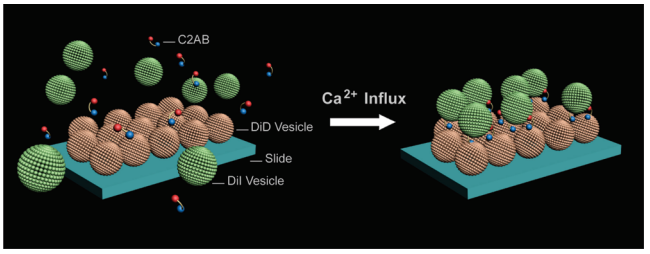

Figure 1. Schematic illustration of the experimental strategy. We immobilized vesicles labeled with DiD and biotinylated lipids on the PEGylated quartz surface via specific binding of biotin and neutravidin, and removed nonimmobilized vesicles by flow washing. We then introduced another group of vesicles, having the same lipid composition but labeled with DiI, along with C2AB into the flow chamber. When we injected $Ca2^{+}$ ions, the DiI-labeled vesicles rapidly bound to the surface-immobilized vesicles using the Ca^{2+}-bound C2AB molecules as the molecular glue. If there was any degree of lipid mixing as a result of vesicle bridging, the FRET efficiency correspondingly increased.

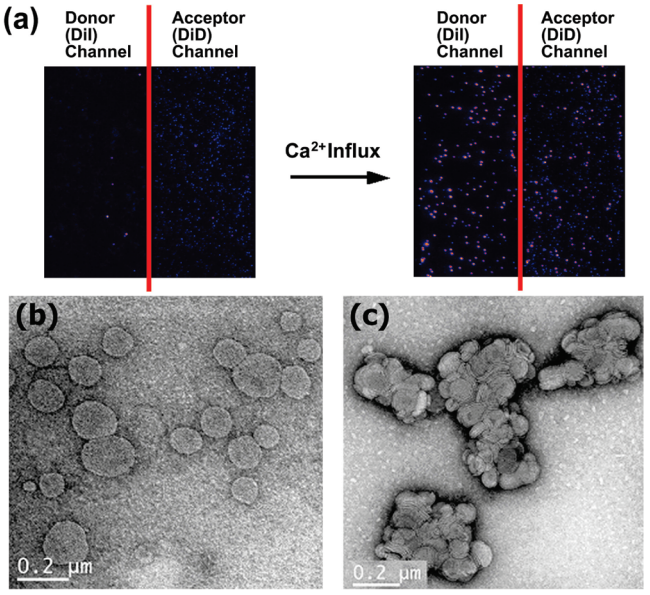

Figure 2. (a) TIR images before and after 0.1 mM Ca^{2+} influx. The images are split into donor and acceptor channels. Only a few fluorescent debris molecules were visible in the donor channel before calcium influx. A large number of bright donor spots in the TIR image appeared shortly after calcium influx indicating the docking of DiI liposomes to the surface-immobilized DiD liposomes. Negative stain transmission electron micrographs of liposomes (35 mol % PS/65 mol % PC) without (b) and with adding 1 μM C2AB and 0.1 mM Ca^{2+} (c).

PC. One group of liposomes was labeled with 2 mol % DiD, a lipophilic fluorophore that emits near 670 nm, and 0.1 mol % biotinylated lipids, while the other liposomes were doped with 2 mol % DiI, another lipophilic fluorophore with emission near 570 nm. The quartz slides were used as substrates for all experiments performed on the home-built total internal reflection (TIR) setup. To eliminate nonspecific binding, all substrate surfaces were processed by the standard PEGylation.[5,14]

The experimental scheme is shown in Figure 1. First, the DiD (acceptor)-labeled liposomes were tethered on the surface via biotin/neutravidin after 15 min incubation at the vesicle concentration of ~100 pM. After washing away the free DiD liposomes, we flowed in a mixture of the DiI (donor)-labeled liposome (~100 pM), C2AB (1 μM), and ethylene glycol tetraacetic acid (EGTA) (10 μM, for elimination of free calcium ions). As shown in Figure 2a, in the absence of Ca^{2+}, we could not see appreciable docking of the DiI-labeled donor liposomes. However, as this mixture was washed out by subsequent injection of 0.1 mM Ca^{2+}, DiI-labeled liposomes began to dock to surface-immobilized, DiD-labeled liposomes rapidly with a time constant of hundreds of milliseconds. Negative stain transmission electron microscopy also verified that the separated liposomes containing 35 mol % PS plus 65 mol % PC clustered together quickly after adding 1 μM C2AB and 0.1 mM Ca^{2+} (Figure 2b and c). The whole process was completed within two seconds (Figure S2 in the Supporting Information).

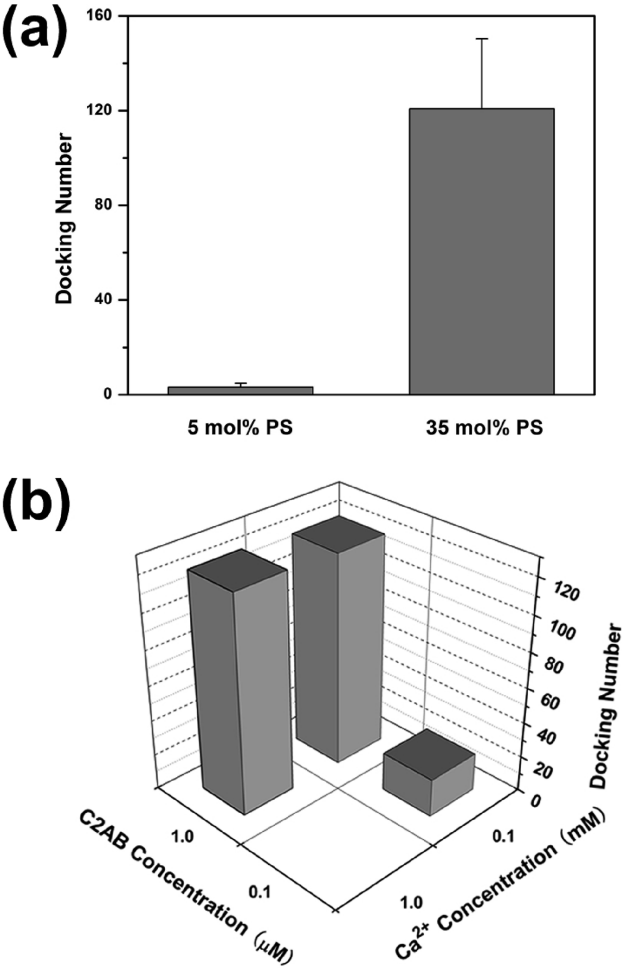

Figure 3. (a) Average number of docked DiI liposomes per imaging area (25 μm×50 μm). We studied two different lipid compositions for vesicles, one with 5 mol % and the other with 35 mol % PS molecules. When the PS concentration was reduced to 5 mol %, the portion of PC lipids was accordingly increased to 95 mol %. A total of 15−20 imaging spots were used to get these average values. Concentrations of 0.1 mM Ca^{2+} and 1 μM C2AB were used. (b) Average number of docked DiI liposomes for various C2AB and calcium concentrations.

Liposome−liposome docking is strongly influenced by the charge−charge interaction. As shown in Figure S1 in the Supporting Information, each C2AB molecule binds up to five calcium ions, and this calcium ion binding greatly increases its affinity for negatively charged lipid membranes, leading to high

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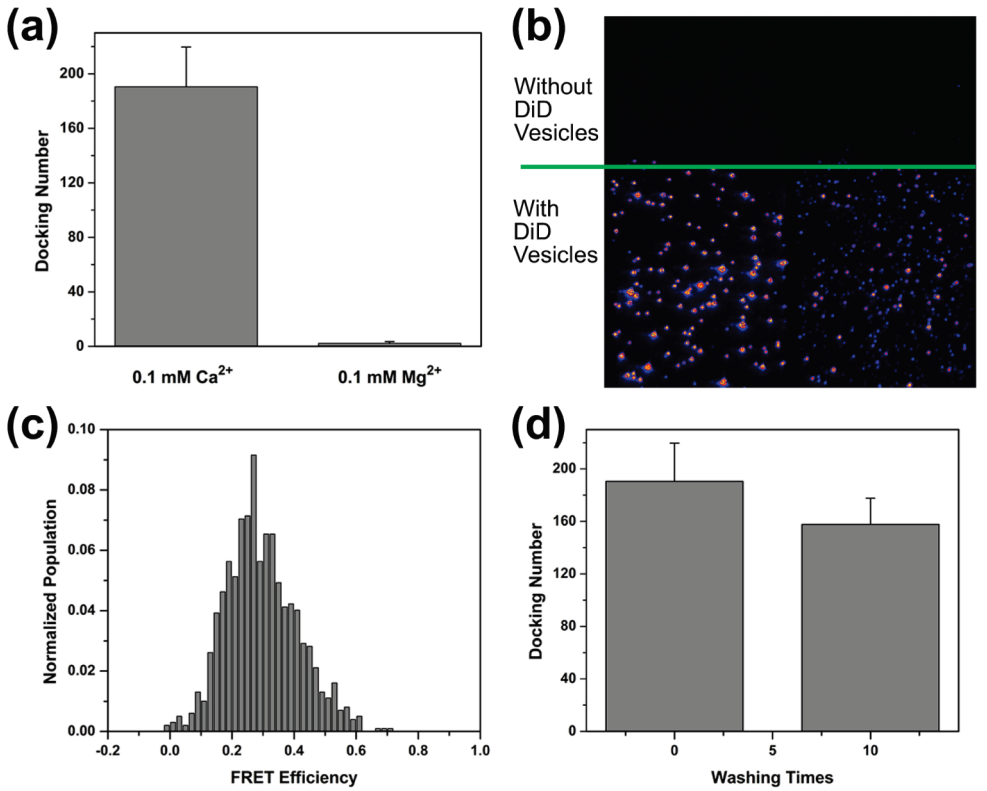

Figure 4. (a) Average number of docked DiI liposomes after 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+} influx. (b) TIR image of a flow channel after 0.1 mM Ca^{2+} influx. The DiD vesicles on the upper part of this flow channel were removed by an air bubble before injecting DiI liposomes. (c) FRET distribution of DiI and DiD vesicle complexes after 0.1 mM Ca^{2+} influx. The FRET population peak at 0.3 represents close-docked state or the hemifusion state of vesicle complexes. (d) Average number of docked DiI liposomes before buffer washing and after 10 rounds of buffer washing, with 150 μL for each round.

local curvatures or a close opposition between two membranes.[12,13] In the presence of the negatively charged lipid PS, the calcium ion affinity of C2AB can be enhanced by 100 times.[15] Therefore, the negatively charged PS molecules play an important role in the interaction between C2AB molecules and calcium ions. When we reduced the PS concentration from 35 to 5 mol %, the gluing of DiI liposomes was extinguished (Figure 3a). Since only five calcium-ion-binding sites are associated with each C2AB molecule, the 0.1 mM Ca^{2+} was saturating for 1 μM C2AB. When the calcium concentration was increased to 1 mM, the docking number of DiI liposomes, which is the number of donor spots in each image area with the size of 25 μm $\times$ 50 μm, remained the same (Figure 3b). Under the calcium-saturated condition, we can control the results by adjusting the amount of C2AB. As shown in Figure 3b, the DiI−liposome docking number was greatly reduced when C2AB concentration was lowered to 0.1 μM.

The effect of the C2AB is specific in terms of the response to calcium ions and the interaction between charged membranes. When we replaced calcium ions with the same concentration of magnesium ions, the glue function of C2AB totally vanished (Figure 4a). This result indicates that this molecular glue is not simply caused by the electrostatic interaction of any divalent ion and specific binding of calcium to C2AB molecules is required.[13] When we partially destroyed a predocked DiD liposome layer by an air bubble before DiI liposomes were injected, as demonstrated in the upper part of Figure 4b, no DiI liposome attachment was observed after calcium washing while the lower part with intact DiD liposomes worked normally. We concluded that no DiI liposome docking was observed without DiD liposomes. This experiment illuminates the specificity of this molecular glue, which hints at wide applicability.

This abrupt and stable appearance of the DiI-labeled liposomes is ascribed to specific docking to DiD-labeled liposomes because the Forster resonance energy transfer (FRET) distribution peaked at a nonzero efficiency value, $E = I_A/I_A + I_D = 0.3$, where I_A and I_D are the sensitized emission intensity of the acceptor and donor, respectively (Figure 4c).[5] This FRET efficiency value strongly suggests that a close packaging state of vesicles, in which the outer leaflets are about to mix, is induced by C2AB and calcium ions. As shown in Figure 2c, this close packaging state is also observed through negative stain transmission electron microscopy. After calcium ion binding, C2AB molecules become positively charged, which has a high affinity with negatively charged lipid molecules of the membrane.[12,13] We found that interaction between C2AB molecules with calcium ions and charged membranes is strong and stable. Even after 10 rounds of buffer washing, with 150 μL for each round, more than 80% of the DiI liposomes still remained bonded on the surface (Figure 4d).

In conclusion, we reported on a two-component molecular glue, C2AB molecules containing calcium ions, specific for negatively charged lipid vesicles. This molecular glue can create a high local concentration of lipid vesicles rapidly, and we anticipate that this super fast, stable, and easy-to-use glue will become a useful tool for liposome applications in biotechnologies and therapeutics. Liposomes have been proposed as biodegradable and essentially nontoxic vehicles for drug delivery. Due to the high local concentration induced by this molecular glue, an

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enhanced therapeutic efficacy would be expected. The liposome with a high concentration of negatively charged lipid molecules, repelling each other from spontaneous fusion in the stock solution and increasing the adhesiveness of the glue, is an ideal partner of this molecular glue for drug delivery purposes. Furthermore, because of this molecular glue, liposomes would be able to work as nanoreactors for the fast *in vivo* synthesis of drug molecules. By using liposomes containing different reactants, we anticipate that, subsequent to clustering driven by C2AB molecules, certain fusogenic agents such as PEG[16,17] and small fusogens[18,19] can be applied to induce full fusion for starting the reaction. In addition, this molecular glue could be useful for controlling the spread of target cells such as tumor cells with negatively charged membranes.[20,21]

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Supporting Information Available: A movie of using the molecular glue, a ribbon diagram of the C2AB molecule, a real-time fluorescent trace with TIR images, and a detailed description of experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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