Anionic Saccharides Activate Liposomes Containing Phospholipids Bearing a Boronic Acid for Ca^{2+}-Dependent Fusion

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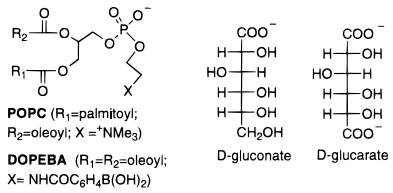
New methods of inducing selective membrane fusion would be useful in hybridoma technology, targeted drug delivery and gene therapy.[1,2] Although a number of macromolecular fusogens are known, there are very few small molecules that can trigger membrane fusion on their own.[2] In certain cases, proteins, peptides, and smaller molecules can induce divalent metal cation-dependent fusion processes. For example, fusion of stripped rough endoplasmic reticulum membranes is mediated by a membrane-bound receptor that is activated by GTP in the presence of Mg^{2+} ions.[3] Here we describe a functional mimic of that general scheme, where liposomes coated with a synthetic boronic acid receptor become susceptible to Ca^{2+}-dependent fusion after they bind anionic saccharides.

Protons and divalent metal cations such as Ca^{2+} are often used to promote the fusion of liposomes containing anionic phospholipids. Association of the anionic head groups with Ca^{2+} ions leads to charge neutralization, head group dehydration, and close membrane apposition, conditions that promote fusion.[2] On the other hand, membranes containing zwitterionic choline phospholipids are much less sensitive to Ca^{2+}-induced fusion. The liposomes used in this study were formed from a 1:1 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and the novel anionic phospholipid DOPEBA, which was prepared



by N-acylating 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine with (4-carboxyphenyl)boronic acid.[4] The vesicles were sized by repeated rapid extrusion through polycarbonate filters with pores of 100-nm diameter.[5] This produced predominately large unilamellar vesicles with an average mean diameter of 99 ± 22 nm as judged by dynamic light scattering. Although vesicles containing certain N-acylated phosphatidylethanolamines are known to become fusogenic in the presence of Ca^{2+},[6] we expected that this tendency would be attenuated if the DOPEBA was mixed at a 1:1 ratio with the lamellar-favoring POPC.[6a,7] Boronic acids are known to spontaneously form reversible chelated complexes with saccharide vicinal-diol groups.[8] Of particular relevance here

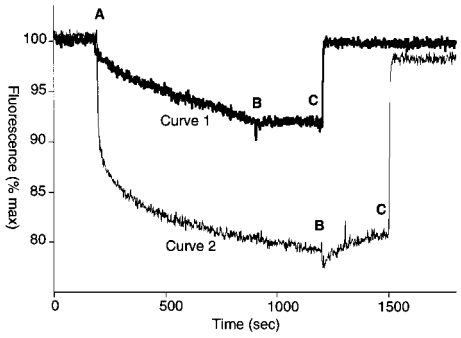


Figure 1. Aqueous contents mixing assay at 25 °C.[13] A population of POPC/DOPEBA liposomes (25 μM) containing ANTS and an equal population containing DPX were mixed in 100 mM NaCl/10 mM MOPS, pH = 7.4, and treated as follows: curve 1, 10 mM sodium gluconate at $t = 0$, 10 mM $CaCl_2$ at A, 20 mM EDTA at B, 15% Triton X-100 at C; curve 2, 7 mM potassium glucarate at $t = 0$, 7 mM $CaCl_2$ at A, 14 mM EDTA at B, 15% Triton X-100 at C.

is the knowledge that borate complexes of polyhydroxycarboxylates are able to sequester Ca^{2+} ions.[9] We hypothesized that monoanionic DOPEBA would combine with anionic saccharides to form multianionic head groups that would associate more strongly with Ca^{2+} ions and increase the propensity for vesicle aggregation and subsequent fusion.

Well-established spectrometric methods were used to measure liposome aggregation (changes in turbidity), leakage (escape of aqueous contents), and fusion (aqueous contents mixing and lipid mixing).[10] Fusion and leakage at 25 °C were examined using the fluorescent ANTS/DPX assays.[11,12] In each experiment, liposome dispersions in 100 mM NaCl/10 mM MOPS, pH 7.4, were treated at $t = 0$ s with small aliquots of concentrated polyhydroxycarboxylate stock solution (pH 7.4) which produced no effect on fluorescence and then $CaCl_2$ was added at $t = 200$ s. The fusion assay started with two equal populations of liposomes (25 μM of each population), one encapsulating the fluorophore ANTS and the other containing the collisional quencher DPX. Fusion and mixing of aqueous contents results in a decrease in ANTS fluorescence intensity. Curve 1 in Figure 1 shows the contents mixing assay for liposomes treated with 10 mM gluconate/$CaCl_2$ which produced 7% fusion at $t = 800$ s.[13] A leakage assay using a single population of liposomes containing coencapsulated ANTS/DPX showed only 3% leakage at $t = 800$

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(4) See the Supporting Information for the preparation and spectral properties of DOPEBA.

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(11) ANTS is 8-aminonaphthalene-1,3,6-trisulfonate, DPX is *N*,*N*′-*p*-xylenebis(pyridinium bromide), NBD-PE is *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phospatidylethanolamine, and Rh-PE is *N*-(lissamine Rhodamine B sulfonyl)phospatidylethanolamine.

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(13) The methods used for calibrating the mixing and leakage assays are described in the Supporting Information. All assays were independently repeated at least once with an average uncertainty of ±10% for all numerical results.

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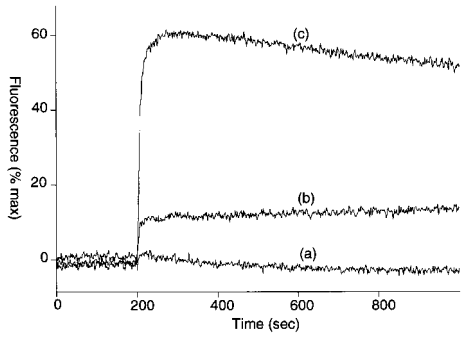

Figure 2. Lipid mixing assay at 25 °C.[13] A population of POPC/DOPEBA liposomes (25 μM) containing 0.3% of each of the probes NBD-PE and Rh-PE was mixed with an equal population of unlabeled POPC/DOPEBA liposomes in 100 mM NaCl/10 mM MOPS, pH = 7.4, and treated at $t = 0$ as follows: (a) no addition; (b) 7 mM sodium gluconate, pH 7.4; (c) 7 mM potassium glucarate, pH 7.4. At $t = 200$ s, all three samples were treated with 7 mM $CaCl_2$.

s. That fusion was occurring to produce larger liposomes was confirmed by light scattering experiments which showed that the turbidity of POPC/DOPEBA liposomes increased after treatment with 10 mM gluconate/$CaCl_2$ at the same rate as contents mixing. In the absence of gluconate or in the presence of 10 mM glucose, the addition of 10 mM $CaCl_2$ induced a negligible change in turbidity and only 3% fusion at $t = 800$ s as judged by contents mixing. Control experiments with liposomes containing only POPC showed no propensity for contents mixing when exposed to 10 mM gluconate/$CaCl_2$.

When the contents mixing assay was repeated with 10 mM glucarate/$CaCl_2$, the POPC/DOPEBA liposomes underwent rapid and extensive fusion concomitant with precipitation and 80% leakage of the aqueous contents. To avoid precipitation, the experiment was repeated with 7 mM glucarate/$CaCl_2$. Curve 2 in Figure 1 shows that, even at the lower concentration, the fusion induced by glucarate is significantly faster and more extensive (19% fusion, 8% leakage at $t = 800$ s) than the gluconate system. In addition, Figure 1 shows that fusion can be arrested by the addition of the Ca^{2+} chelator EDTA and that lysis of the liposomes with Triton X-100 releases the mixed encapsulated contents.

Fusion was also monitored by the fluorimetric probe dilution assay which uses the fluorescently labeled phospholipid NBD-PE and its resonance energy transfer quencher Rh-PE.[11,14] Two equal populations of POPC/DOPEBA liposomes were mixed at $t = 0$ s along with polyhydroxycarboxylate. One liposome population contained 0.3% of each the probes NBD-PE and Rh-PE, whereas the other population was unlabeled. Lipid mixing is indicated by an increase in NBD-PE fluorescence intensity due to diminished quenching as the two probes are diluted. The probe dilution assay is considered to be insensitive to liposome aggregation.[14] As shown in Figure 2, there was very little lipid mixing when the POPC/DOPEBA liposomes were treated with 7 mM $CaCl_2$ in the absence of polyhydroxycarboxylate. In the presence of 7 mM gluconate, there was about 10% mixing of lipids when $CaCl_2$ was added at $t = 200$ s, whereas in the presence of 7 mM glucarate. the lipid mixing was around 60%.[13,15]

The results clearly indicate that on its own polyhydroxycarboxylate or Ca^{2+} has little effect on anionic POPC/DOPEBA liposomes, whereas the synergistic combination of polyhydroxycarboxylate and Ca^{2+} induces liposome aggregation and fusion.[16] Moreover, the combination of glucarate/$CaCl_2$ is significantly more effective than gluconate/$CaCl_2$. The most likely explanation is that the DOPEBA combines with the gluconate to form a trianionic phosphoboronate head group which can homodimerize intra- and/or intermolecularly via a bridging Ca^{2+}. In the case of glucarate, the corresponding tetraanionic phosphoboronate can associate more strongly with the Ca^{2+}, possibly producing oligomeric domains at the vesicle contact point (Figure 3) which may destabilize the aggregated vesicles and/or stabilize the fusion intermediates.[10]

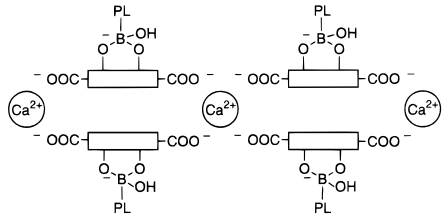

Figure 3. Stylized representation of putative oligomeric domain formed by DOPEBA/glucarate/Ca^{2+} at the vesicle contact point. PL = phospholipid.

Most recently we have used the lipid mixing assay to measure the ability of sugar phosphates to induce Ca^{2+}-dependent fusion of POPC/DOPEBA liposomes. We find that at 10 mM fructose 6-phosphate, fructose 1,6-diphosphate, and uridine 5′-monophosphate all have little or no effect on lipid mixing. However, the 5′-di- and -triphosphate derivatives of uridine are increasingly effective at inducing Ca^{2+}-dependent fusion.[17]

Our findings are significant for a number of reasons. In terms of drug delivery, the technical issues of prolonged liposome lifetime and liposome targeting have recently been improved; however, the problem of inefficient cell transfection remains to be solved.[1,18] While there are a number of methods known to trigger liposome release,[19] there are very few ways of triggering fusion and contents mixing.[20] The liposome system reported here is the first that can be activated for Ca^{2+}-dependent fusion by treatment with nontoxic anionic saccharides.[16] This raises the possibility of a new approach to drug or reagent delivery using "sugar-sensitive liposomes" (i.e., liposomes that can be selectively triggered, by a high local dose of anionic saccharide, to undergo fusion and cell transfection). From the broader perspective of supramolecular chemistry, our results in combination with others[10c,21] suggest that an effective way to design a membrane fusing system is to use molecular recognition to induce the formation of noncovalent oligomeric domains at the membrane contact point.

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Supporting Information Available: Text describing DOPEBA synthesis, liposome preparation, and fusion assays and an ^1H NMR spectrum of DOPEBA (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(15) Reverse addition experiments were also conducted. At $t = 0$, 7 mM $CaCl_2$ was added which produced negligible lipid mixing, then 7 mM glucarate was added at $t = 200$ s which induced 65% lipid mixing in a few seconds. This result implies that boronate complex formation must be fast (k_{on} less than seconds), which agrees with previous studies.[8]

(16) The extent of Ca^{2+}-induced fusion observed with our three-component POPC/DOPEBA/polyhydroxycarboxylate system is similar to that observed with anionic liposomes having related compositions (e.g., PC/PS,[7a] PC/PA,[7b,10b] or PC/*N*-acyl PE[6a]). Preliminary lipid mixing assays indicate that POPC/DOPE/DOPEBA (2:3:3) liposomes can be induced to fuse with POPC liposomes.

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