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Embedding resorcinarene cavitands in lipid vesicles†

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Abstract

A fluorescently labeled resorcinarene cavitand has been successfully embedded in DLPC lipid vesicles and imaged using confocal microscopy. The cavitand resides exclusively in the bilayer.

Resorcinarene cavitands are deep, highly ordered supramolecular species that have a proclivity for small molecule guest binding in organic solution.¹ Alterations to the feet² and rim^{3,4} have afforded water soluble species. More recently, several cavitands have been shown to retain their function as small molecule binders^{5,6} and as pH responsive molecular switches⁷ while embedded in aqueous micelles. In addition to micelles, lipid bilayers have also proven to be compatible; guests appended with fluorophores and proteins bind to resorcinarenes as determined by surface plasmon resonance.⁸ With the cellular uptake of calixarenes demonstrated,⁹ resorcinarenes are sure to follow. Micelles provide an excellent environment for characterizing host–guest behavior using NMR techniques. The larger size of vesicles and more ordered lipid bilayer environment provide additional methods of characterization and more importantly provide a better model for the cell. In this report we demonstrate a method suitable for embedding and visualizing resorcinarene cavitands in the lipid interior of phosphocholine vesicles. This work sets the stage for visualizing and quantifying host guest binding events in a lipid bilayer environment.

To better understand their behavior in lipid bilayer environments we prepared a fluorescent resorcinarene cavitand. We had several key criteria in mind with our design: (1) an ideal cavitand should have significant hydrophobic character so that it partitions into the bilayer, (2) it should be amenable to fluorescent tagging, and (3) should retain its ability to serve as a small molecule host after chemical modification and embedment.

Monofunctionalized resorcin[4]arene **1** was readily prepared by a literature method from the condensation of resorcinol, hexanal, and 2,3-dihydrofuran (Fig. 1).¹⁰ We then transformed the shallow resorcinarene into a cavitand through addition of four difluorodinitrobenzenes. Conversion of the eight nitro groups to amines followed by *in situ* condensation with propionyl chloride gave an octaamide that possessed one free hydroxyl for further transformation.^{1,11} We next transformed the alcohol to a mesylate with mesyl chloride then to azide using sodium azide. Copper iodide with tris-(benzyltriazolylmethyl)amine (TBTA) catalyzed triazole formation was straight forward using propargyl amine appended nitrobenzoxadiazole (NBD) as our fluorophore coupling partner giving **3** in 64% yield from azide **2** (see ESI for full experimental details†).¹² ¹H NMR and high-resolution mass spectrometry are fully consistent with the assigned structure.

†Electronic supplementary information (ESI) available: Full synthetic procedures and characterization of all new compounds is provided. Additional confocal images and full z-scan analysis is included. See DOI:10.1039/c2nj20961f

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Having prepared our fluorescent cavitand we next tested its ability to bind small molecule guests. Mixing **3** with adamantyl amine in mesitylene-*d*₁₂ gave rise to characteristic upfield signals associated with guest encapsulation¹ (see ESI†). Similar results were not seen in aqueous micelles. The reasons are not known at this time—an analogous tetra ethyl footed cavitand did bind guests in aqueous micelles.⁵ The functionality of **3** in water has not been fully explored, but by changing the feet or rim this issue can be resolved. Having established the cavitand as an active host in organic solvents we turned our attention to developing a protocol for visualization when in an aqueous vesicle environment.

Many methods exist for the preparation of giant unilamellar vesicles (GUVs) that are suitable for optical characterization.¹³ One method that has been underutilized in our opinion is incredibly flexible and affords polydisperse GUVs in 4 min.¹⁴ Characterization of the bilayer of these vesicles is simple using phase contrast and confocal microscopies.¹⁵ In short this method entails: (1) lipid(s) dissolved in chloroform and methanol are suspended in an aqueous buffer solution; (2) desired additives are introduced; (3) the suspension is rotavapped at 40 °C for 4 min to remove all organic solvents, and optionally (4) gel permeation or dialysis can be applied to remove extravesicle additives. The samples are then placed on inverted deep well microscope slides and examined at 10–40 × magnification using confocal microscopy.

Hydrophilic small molecule fluorophores are not expected to partition into the membrane bilayer of DLPC vesicles. We prepared 2-(Thioureido fluorescein)ethyltrimethylammonium Chloride (**4**) by condensation of fluorescein isothiocyanate and 2-aminoethyltrimethylammonium chloride following literature procedure.⁸ When **4** was added to a solution of DLPC vesicles, the aqueous exterior is fluorescent and the vesicle interior is dark (Fig. 2A). When **4** was added to the lipid solution prior to vesicle formation, fluorescence is observed both inside and outside of the vesicle and the lipid bilayer is notably darker (Fig. 2B). Size exclusion chromatography or dialysis removes the external dye giving dye-loaded vesicles (Fig. 2C). These control experiments illustrate the behavior of hydrophilic fluorophores—they reside exclusively in the aqueous environment. The same results were observed for carboxyfluorescein (See ESI†).

Hydrophobic molecules on the other hand would be expected to behave differently. When cyclohexylamine-NBD was added to a vesicle solution (before or after vesicle formation) we observe migration of the dye into the lipid bilayer (Fig. 3A). Confocal microscopy z-scan analysis illustrates the location of the dye when imaged at half the height of the vesicle. To confirm this result we prepared vesicles and incorporated 1 mole% 1-acyl- 2-(12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl)-*sn*glycero- 3-phosphocholine prior to vesicle formation. Again the lipid bilayer is fluorescent (Fig. 3B). With cyclohexylamine NBD we noted that many multilamellar vesicles and other very small species were formed.

Having established the visual controls necessary to differentiate between the localization of hydrophilic and hydrophobic fluorescent molecules we next examined the behavior of resorcinarene **3**. Cavitand **3** (1 mol%) was introduced to lipid prior to vesicle formation. Removal of organic solvents resulted in the formation of viable vesicles. Z-scan analysis at several heights (Fig. 4) reveals fluorescence exclusively in the lipid bilayer.

In the first frame we see two vesicles at their apex. Moving 2 microns in the z-dimension the bilayer is revealed, and after another 2 we arrive at the half-height of the upper vesicle. In the final frame have arrived at the bottom of the upper vesicle, but not quite at the lower

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vesicle, which is larger in size (See ESI for all frames and corresponding DIC images†). This result is consistent with the results for hydrophobic species seen in Fig. 3. Cavitand **3** is convincingly located in the lipid bilayer.

We anticipate that most resorcinarene cavitands will have the same behavior due to their significant hydrophobic surface. Based on work with lipid bilayers on SPR (surface plasmon resonance) chips, the water soluble cavitand with ethyl feet and four carboxylates displays consistent results.⁸ The incorporation of resorcinarenes into lipid bilayers and their host guest binding, along with our report sets the stage to study other pressing issues with these synthetic receptors. Microscopy is now an additional tool to study events in these larger ensembles where NMR spectroscopy is no longer the most easily applicable technique. The binding of small molecules in micelles,^{5,6} the binding of proteins to surfaces with embedded cavitands,⁸ two component switching,⁷ and ion sensing¹⁶ are all recent first inroads of applying resorcinarenes to biological model systems and problems. We believe that resorcinarene cavitands have even more potential as controllable optical storage devices, molecular machines and may provide other exciting opportunities for applications to biological problems. We will report our findings on some of these points in due course.

Experimental procedure for vesicle preparation and visualization

1,2 dilauroyl-sn-*glycero*-3-phosphocholine (15 μ L, 0.0804M in chloroform, Avanti® Polar Lipids), methanol (200 μ L), chloroform (1 mL), and Hepes (3 mL, 0.01 M in 18.2 M H_2O) were placed in a 50 mL pear-shaped flask. The solution was slowly rotovapped at 10 mmHg, 40 °C, 40 rpm for 4–5 min.¹⁴ After two distillation events were observed an opaque liquid resulted. When using cavitand **3**, 1 mol% was added prior to vesicle formation. When using fluoresceincholine **4**, 2.5 mol% was added prior to or post vesicle formation. When using carboxyfluorescein, 5 mol% was added prior to or post vesicle formation (see ESI†). For gel filtration we employed Bio Rad Econo-Pac® 10 DG 10 mL Disposable Chromatography Columns and for dialysis Spectra/Por® Dialysis Membrane, MWCO: 6–8000, Nominal Flat Width: 10 mm, Diameter: 6.4 mm, Vol/Length: 0.32 mL cm^{-1} , Lot # 3248269, using a minimum of 3 buffer exchanges. Deepwell slides were prepared using 80 μ L of GUV solutions. Slides were viewed on an inverted Olympus Fluoview 1000 confocal laser scanning system utilizing a Olympus IX-81 microscope with a 40x oil objective and 2x digital zoom. The dyes were excited by a 488 nm argon ion laser, and emission was captured at 559 nm. The dimensions of the images taken were 1024 $\times$ 1024 pixels and a z-scan analysis was performed capturing a minimum of 10 planes. On average vesicles imaged were 10 $\pm$ 2 microns in diameter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Synthesis of fluorescent monoNBD functionalized octaamide cavitand **3** and MMFF rendition (see ESI for full details†).

Fig. 2. Fluorescent confocal images (Olympus Fluoview 1000, IX-81; ex: 488; em 559; 40 ×) of prepared DLPC vesicles that were then treated with **4** (A), DLPC vesicles that were prepared with **4** present prior to vesicle formation (B), and that were then subjected to dialysis (C). Vesicles are approximately 10 microns. For DIC images see ESI†

Fig. 3. Fluorescent confocal images (Olympus Fluoview 1000, IX-81; ex: 488; em 559; 40 X) of prepared DLPC vesicles that were treated with 1% cyclohexylamine-NBD (A), and DLPC vesicles that were prepared with 1% 1-acyl-2-(12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-dodecanoyl)-*sn-glycero*-3-phosphocholine (B). Vesicles are approximately 10 microns. For DIC images see ESI†

Fig. 4. Fluorescent confocal images (Olympus Fluoview 1000, IX-81; ex: 488; em 559; 40 X; z-scan analysis) of prepared DLPC vesicles that had 1 mol% of cavitand **3** present at four different heights in the z-dimension (heights listed). Vesicles are approximately 10 microns.