Supporting Information

Single Molecule Nanocontainers Made Porous Using a Bacterial Toxin

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Vesicle-to-Vesicle Variation of Effective Reagent Concentration in the Absence of Pores

The number of reagents encapsulated in a sealed vesicle will likely follow a Poisson distribution\(^1\). For a particular concentration, there will be a finite number (N) of a particular reagent inside vesicles on the average and this number will vary with the standard deviation of \(\sqrt{N}\) among individual vesicles. For example, if Mg\(^{2+}\) concentration is 30 \(\mu\)M for a 100 nm diameter vesicle, N is \(~10\) and the actual effective concentration can vary with about 30\% relative standard deviation around 30 \(\mu\)M which could mean a significant difference in the local environments for the encapsulated molecules. In addition, the heterogeneity in vesicle size would also contribute to a variation in the intravesicular reagent concentration if no pores exist on vesicles.

Rep Helicase Encapsulation

Separation of Unencapsulated Rep. A Bio-Rad column was packed with 2mL of Ni-NTA bead suspension. After adding the Ni-NTA beads, the column was first washed with 3mL of distilled water, then \(~10\)mL of buffer R (see main text, Materials and Methods). The column material was thus equilibrated with the buffer in which the vesicles were prepared. Having the beads all settled down, \(~150\)\(\mu\)L of the sample was gently loaded on the Ni-NTA bead matrix. Initially, 3-4 drops
(volume of one drop was determined to be typically ~50µL) came out of the column due to the volume displaced by the sample loading. After waiting 5 min. to make sure that the binding to column is equilibrated, 3mL of buffer R was used to elute the sample. The separation was first characterized using solutions of Rep or vesicles only. We found that up to 1µM of Rep could totally be captured by the Ni-NTA column (data not shown), and the vesicles eluted between ~10-15th drops. For the Rep encapsulation sample, drops between 10-15 (counting includes the first 3-4 drops during the sample loading) was collected in a test tube, and used immediately.

*The molecules are successfully encapsulated inside vesicles.* Vesicles were diluted by 20 to 200 times in T50 buffer (10mM Tris pH 8.0, 50mM NaCl) and then incubated with the coated quartz slide. PEG coated slides were used for the Rep experiments. When the slide surface was not covered with neutravidin, insignificant binding was observed. In sharp contrast, same dilution of vesicles yielded a good coverage of fluorescent spots on the surface after neutravidin incubation (Supporting Figure 3). We therefore concluded that the binding was specific to only the biotinylated objects (i.e. the vesicles), and that the non-specific binding was undetectable.

*Rep Helicase Does not Interact with Lipid Membranes.* The headgroup of EggPC lipids which were used for making the encapsulation samples is zwitterionic. Although there is no particular reason to think that the Rep helicase would interact with the lipids, we tested the binding of Rep helicase directly on SLB or blank vesicles immobilized on SLB. 1nM of Rep showed very few binding the surfaces (data not shown) provided that a high quality SLB is formed. Although such measurements cannot rule out transient interactions between Rep and lipid membranes, evidently the Rep activity is not altered inside vesicles.

*Skipping Freeze-Thawing Restores Rep Activity.* The freeze-thaw cycle is not essential but is thought to make encapsulation more efficient. We had previously characterized the effect of freeze-thawing on the unwinding activity of Rep helicase at the bulk level. 10 freeze-thaw cycles decreased the DNA unwinding amplitude by only 5% (data not shown) when the thawing was carried out in room temperature water bath. However, single molecule measurements done on vesicles prepared with freeze-thawing showed no Rep activity (translocation). We therefore skipped the freeze-thawing step during the encapsulation only after when Rep translocation was observed for encapsulation samples. The distribution of the number of encapsulated molecules was not uniform, i.e. some vesicles had only one Rep molecules whereas others had multiple
(e.g. four or five of them) encapsulated within (The number of Rep molecules could be estimated by counting the digital photobleaching events.). Such non-uniform encapsulation efficiency could be due to aggregation of Rep monomers or reflect the fact that the freeze-thawing makes the encapsulation not only more effective, but also more homogenous. For the single molecule analysis, only the traces that showed proper signatures - e.g. single step photobleaching, and constant total (donor + acceptor) intensity - were considered (Supporting Figure 4).

**Number of aHL Pores Required for Rapid Buffer Exchange**

Although we did not quantify the average number of pores per vesicle, a previous theoretical model of how the permeation rate varies with the occupancy of pores on a membrane surface can provide sensible estimation\(^2\) about the number of pores required for reasonable buffer exchange rates. The diffusion current (I) into a non-adsorbing sphere (of radius R) with disc-shaped pores (of radius r each) on its surface is estimated as:

\[
I = I_{max} \cdot \left(1 + \frac{\pi R}{N r}\right)^{-1}
\]

Here N is the number of pores and \(I_{max}\) is the maximum current that would have flowed if the surface of sphere had been totally hollow. As can be seen in Supporting Figure 5, it takes a relatively low surface coverage for the pores to achieve significant fluxes. For instance, only \(~30\) aHL pores on a 200nm diameter vesicle surface would suffice to absorb the extravesicular agents 10% as rapidly as the maximally crowded aHL pores (i.e. hypothetically \(~1600\) pores). Hence a good exchange rate can be achieved by a relatively low number of pores without disrupting the intactness of the vesicle in principle and, it is therefore no surprise that sensible exchange rates can be achieved without jeopardizing the integrity of the vesicles.

**References:**

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Supporting Figure 1. Dwell time analysis of the RNA four-way junction in 50mM Mg$^{2+}$, measured in vesicles. Each open circle represents the average dwell times in the high and low FRET states determined from individual molecules encapsulated within vesicles. Encapsulated junction construct is schematically depicted on the upper right-hand corner. The junction inside the vesicle displays a similarly distributed lifetimes of the two states for individual molecules compared to the data presented in a previous paper (Tan et al, Proc Natl Acad Sci U S A. 2003, 100, 9308-13), and that their average lifetimes determined at 50 mM Mg$^{2+}$ inside the porous vesicle are, as expected, slightly shorter than those obtained at 100 mM Mg$^{2+}$ from surface-immobilized molecules also reported in Tan et al.
Supporting Figure 2. Hemolysis assay for recombinant aHL prepared in our lab. The rabbit red blood cells (rRBC) were re-suspended in an isotonic PBSA buffer (PBS buffer with 1mg/ml BSA), and briefly centrifuged after 30min. The absorbance for the supernatant was measured. (Left) In the absence of aHL, the absorbance is similar to that of the (PBSA) buffer only. In the presence of aHL, the supernatant exhibited absorption spectrum which is a reminiscent of the hemoglobin released in solution. Hemoglobin release happens due to the lysis of rRBCs by aHL. (Right) Titrations over various aHL concentrations. 100% lysis is achieved by diluting rRBCs in distilled and de-ionized water or a detergent solution. The data is in good agreement with previous findings (It is well known that it takes about 1nM to lyse rRBC, whereas much higher concentrations are required for human erythrocytes or artificial liposomes\(^3\)). The line is drawn as a guide to the eye.
Supporting Figure 3. Specific attachment of vesicles encapsulating molecules of Rep and DNA. Same concentrations of Ni-NTA column treated vesicle samples were incubated with the surface before (left) and after (right) neutravidin attachment. Binding was thus highly specific to encapsulated molecules because only the vesicles contained biotin.
Supporting Figure 4. Donor (light gray) and acceptor (dark gray) intensity trace of FRET data shown in Figure 3c. Single step photobleaching of the acceptor signal indicates that the observed shuttling was from a single Rep monomer. Not shown is the later occurring single step photobleaching of the donor signal, hence single DNA.
Supporting Figure 5. Equation (1) plotted for aHL pores (r=0.9nm) on 200nm diameter (R=100nm) vesicles. The parameters for the radii of aHL pores and the vesicles are assigned by the experimentally measured values (by X-ray crystallography and dynamic light scattering respectively). Y axis is the normalized flux ($I/I_{\text{max}}$) where X axis resembles the number of pores on the membrane (N). Half maximum flux can be achieved by ~350 pores.