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High Affinity Host–Guest FRET Pair for Single-Vesicle Content-Mixing Assay: Observation of Flickering Fusion Events

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Supporting Information

ABSTRACT: Fluorescence-based single-vesicle fusion assays provide a powerful method for studying mechanisms underlying complex biological processes of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor)-mediated vesicle fusion and neurotransmitter release. A crucial element of these assays is the ability of the fluorescent probe(s) to reliably detect key intermediate events of fusion pore opening and content release/mixing. Here, we report a new, reliable, and efficient single-vesicle content-mixing assay using a high affinity, fluorophore tagged host–guest pair, cucurbit[7]-uril-Cy3 and adamantane-Cy5 as a fluorescence resonance energy transfer (FRET) pair. The power of these probes is demonstrated by the first successful observation of flickering dynamics of the fusion pore by *in vitro* assay using neuronal SNARE-reconstituted vesicles.

The fusion of vesicles—to other vesicles, organelles, or the plasma membrane—plays a crucial role in cellular reactions, such as vesicle trafficking, exocytosis and neurotransmitter release.[1] A majority of synaptic vesicle fusion is mediated by SNAREs, which act as molecular tethers between the two fusing bodies. Decades of research have been devoted to uncovering the mechanisms of fusion by SNAREs and the roles of regulatory proteins, including synaptotagmins, complexins, and SM (Sec1/Munc18) proteins.[2] Due to the inherent complexity, however, many details of this process continue to be debated.[2a,b,3] Defining the physiological interrelation between stimulation and specific steps in the synaptic exocytosis pathway is crucial to understanding the molecular mechanisms of the synaptic vesicle fusion.

In vitro fusion assays using SNARE-reconstituted vesicles have played a vital role in elucidating the mechanisms of SNARE-mediated vesicle fusion by allowing controlled observations that are not possible by *in vivo* studies.[4] Single-vesicle fusion assays, in particular, enable measurement of fusion dynamics and intermediate stages of fusion[5] that might otherwise not be detectable using ensemble measurements.[2c,5a] A common version of single-vesicle fusion assays uses lipid mixing, which measures the exchange of lipids upon bilayer fusion.[4a,c,5a,6] There is a growing recognition, however, that in spite of the general assumption that the extent of lipid mixing between vesicles is correlated with the extent of content mixing, a high level of lipid mixing can occur without content mixing.[4c,7] To address this concern, fluorescent probes that directly measure the release/mixing of content molecules have been reported recently.[4c,7b,8] However, it is hard to discriminate possible intermediate states between initiation of pore opening and full fusion by these methods. Furthermore, they can also be blind to detection of fusion pore dynamics which may regulate the amount and the rate of vesicle cargo release.[9] Thus, a content mixing assay with high sensitivity that allows an efficient detection of SNARE-mediated vesicle fusion pore dynamics is still called for.

Here, we introduce a novel fluorescence resonance energy transfer (FRET) sensor based on the host–guest pair cucurbit[7]uril (CB[7]) and adamantylamine (Ad) that combines the benefits of small molecule with the high signal-to-noise ratio (SNR). We and others reported that CB[7], a member of the cucurbit[*n*]uril (CB[*n*], *n* = 5–8, 10, 14) host family[10] with a hydrophobic cavity and two identical carbonyl-fringed portals, forms a stable 1:1 host–guest complex with ferrocene or adamantane derivatives in aqueous solution with an exceptionally high binding affinity ($K_a \approx 10^{12}$ and 10^{14} M^{-1}).[11] The unique features of these host–guest complexes have been exploited in biological applications, such as supramolecular fishing of membrane proteins.[12] We envisage that the highly specific and ultrastable synthetic host–guest pair, CB[7]-Ad, could be useful for content-mixing assays. To achieve this goal, we synthesize CB[7] conjugated with a donor dye Cy3 (CB[7]-Cy3) and Ad conjugated with an acceptor dye Cy5 (Ad-Cy5). When vesicles, each containing either CB[7]-Cy3 or Ad-Cy5, fuse, the mixing and binding of the host–guest pair result in the emergence of FRET signal, which is monitored by total internal reflection fluorescence microscope (TIRF) (Figure 1). Remarkably, we observe, for the first time, the fusion pore flickering events, i.e., a repetitive opening and closure of the fusion pore in an *in vitro* content mixing assay and measure the pore opening rate and the dwell time of pore closure state.

We synthesized the fluorophore-tagged CB[7]-Cy3 and Ad-Cy5, after confirming that the optimized structure of the complex is appropriate for FRET by molecular mechanics modeling (Figure S1). Photoreaction of monoallyloxy-functionalized CB[7][13] and cysteamine hydrochloride afforded monoamino-functionalized CB[7] (Figure S2), which was then treated with *N*-hydroxysuccimidyl-Cy3 to produce CB[7]-Cy3 (Figure 2a).

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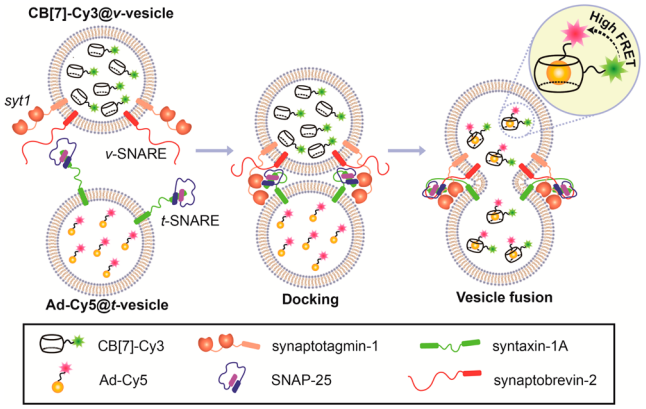

Figure 1. Schematic illustration of SNARE-mediated content mixing using a host–guest FRET pair. *v*-vesicle containing CB[7]-Cy3 and *t*-vesicle containing Ad-Cy5 undergo fusion process: vesicle docking by SNARE proteins and vesicle fusion with content mixing. Content mixing is detected by a FRET signal generated by the host–guest binding between CB[7]-Cy3 and Ad-Cy5.

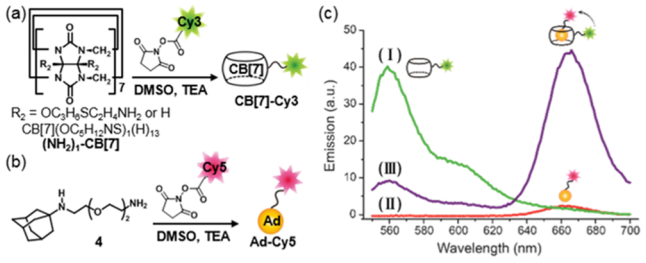

Figure 2. Synthesis and characterization of monodye-conjugated CB[7]-Cy3 and Ad-Cy5 FRET pair. Synthetic scheme of (a) CB[7]-Cy3 and (b) Ad-Cy5. (c) Emission fluorescence spectra of (I) CB[7]-Cy3 only (0.9 μM, green line), (II) Ad-Cy5 only (0.9 μM, red line), and (III) 1:1 mixture of CB[7]-Cy3 and Ad-Cy5 (each 0.9 μM, purple line). All spectra were obtained by 530 nm excitation in a HEPES buffer.

To synthesize the FRET counterpart, amine tethered adamantane **4** was first prepared in a three-step synthesis (Figure S3). A subsequent nucleophilic reaction of **4** with an activated ester derivative of Cy5 resulted in Ad-Cy5 (Figure 2b). Both CB[7]-Cy3 and Ad-Cy5 were purified by HPLC and the purified compounds were thoroughly characterized before use (Figure S4–10). We confirmed that these dye-conjugated CB[7]-Ad pairs produced high FRET signals in aqueous solution upon host–guest binding (Figure 2c). By contrast, in control experiments using nonlabeled host or guest molecules, almost no FRET signal was detected (Figure S11).

A classic *in vitro* assay for neuronal vesicle fusion consists of a *v*-vesicle, which is embedded with the proteins *v*-SNARE VAMP-2 (a.k.a. synaptobrevin-2) and synaptotagmin-1 (*syt1*), and a *t*-vesicle, which is embedded with a *t*-SNARE (a heterodimer of syntaxin-1A and SNAP-25) (protein/lipid = 1:500 for *t*-SNARE or VAMP-2 and 1:900 for *syt1*).[4a,7a] CB[7]-Cy3 and Ad-Cy5 could readily be encapsulated inside the *v*- and *t*-vesicles, respectively (to produce CB[7]-Cy3@*v*-vesicle and Ad-Cy5@*t*-vesicle). The average diameter of the proteoliposomes was ~80 nm (Figure S12). The diffusion coefficients of the probes encapsulated inside the vesicles were much smaller than those of free probe molecules or physical mixture of probe molecules with vesicles, confirming successful encapsulation of probe molecules inside the vesicle without binding directly to the lipid membrane (Figure S13). Using single-vesicle photobleaching measurements (Figure S14), we estimated the number of content molecules encapsulated in a vesicle, typically prepared in the presence of 400 μM content molecules, to be approximately 40. We also confirmed the lack of probe leakage from the vesicles during fusion process (Figure S15).

To test whether our FRET pairs can successfully detect vesicle fusion, we performed a surface-immobilized single-vesicle content-mixing assay.[4c,5] First, Ad-Cy5@*t*-vesicles were immobilized on the surface of a quartz slide coated with PEG through biotin-neutravidin interactions. A 100 pM solution of CB[7]-Cy3@*v*-vesicles was then injected into the flow chamber at 37 °C. Using TIRF microscopy, Cy3 and Cy5 fluorescence signals from the single vesicles were recorded with 50 ms resolution (Figure S16). Docking of a donor *v*-vesicle onto the acceptor *t*-vesicle occurred as evidenced by the sudden appearance of Cy3 emission in the time trace (I_D in Figure 3a). After a few seconds, a

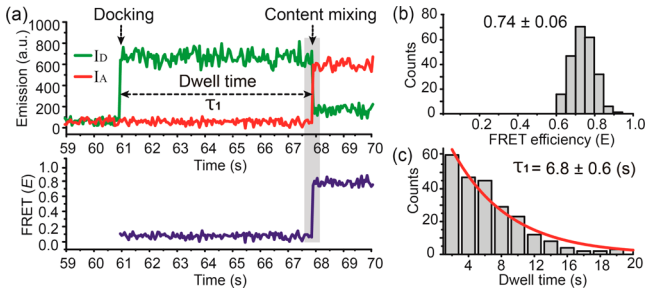

Figure 3. (a) Trajectory of single-step content mixing event. Green and red lines denote Cy3 emission (I_D) and Cy5 emission (I_A), respectively. Blue line represent FRET efficiency ($I_A/(I_D + I_A)$). (b) Distribution of FRET efficiency of each fused vesicle after reaching steady state, i.e., full fusion. The mean value of FRET distribution is 0.74 ± 0.06. (c) Distribution of dwell time (τ_1) between vesicle docking and initial fusion, fit to single exponential decay (red line, $y = A \exp(-x/\tau_1) + y_0$), where τ_1 is 6.8 ± 0.6 s.

rapid increase of Cy5 emission, i.e., a FRET signal (I_A) was observed (Figure 3a). The observed dynamics are consistent with the known mechanism of SNARE-mediated fusion: the SNARE proteins, i.e., *t*-SNARE (on the plasma membrane) and *v*-SNARE (on the synaptic vesicles)[2] initially interact to dock the two vesicles. Subsequent formation of a tight parallel four-helix bundle by the *t*- and *v*-SNAREs brings the two opposite membranes into a close proximity, which triggers membrane fusion and pore opening. The pore opening leads to mixing of the content molecules of the two opposite vesicles to form a strong host–guest complex, which generates a rapid increase of I_A. The average FRET efficiency value (E) determined from hundreds of fused vesicles was 0.74 ± 0.06 (Figure 3b). By comparison, when we encapsulated a 400 μM mixture of CB[7]-Cy3 and Ad-Cy5 into *t*-vesicles, the average E value observed was 0.80 ± 0.04 (Figure S17). This suggests that the E value obtained by the content-mixing assay (~0.74) is a reasonable indicator of full fusion between the two vesicles.

To confirm the negligible effect of encapsulated content molecules on the fusion process, we compared the population-wide fusion rates and efficiencies as measured by our FRET sensor with those measured using Sulforhodamine B (SRB) and found no difference in these values (Figure S18). The dwell times between vesicle docking and fusion pore formation (τ_1) in both assays were identical (Figure 3c and Figure S19). Also, no significant difference in fusion efficiency was observed at various

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concentration of our content molecules encapsulated in vesicles (Figure S20).

In vesicle fusion assays, the power to resolve the intermediate states between unfused and fused states is limited by SNR of the probing signal. We quantified SNR and found that measurements with our host–guest FRET pair had an SNR of ~19, while SRB had an SNR of only ~5 (Figure S21). The SNR for SRB calculated from our experiments is consistent with the SNR of SRB estimated from published data.[4c,7b] If the fusion pore flickering occurs in a vesicle pair (Figure 4a), a multiple stepwise

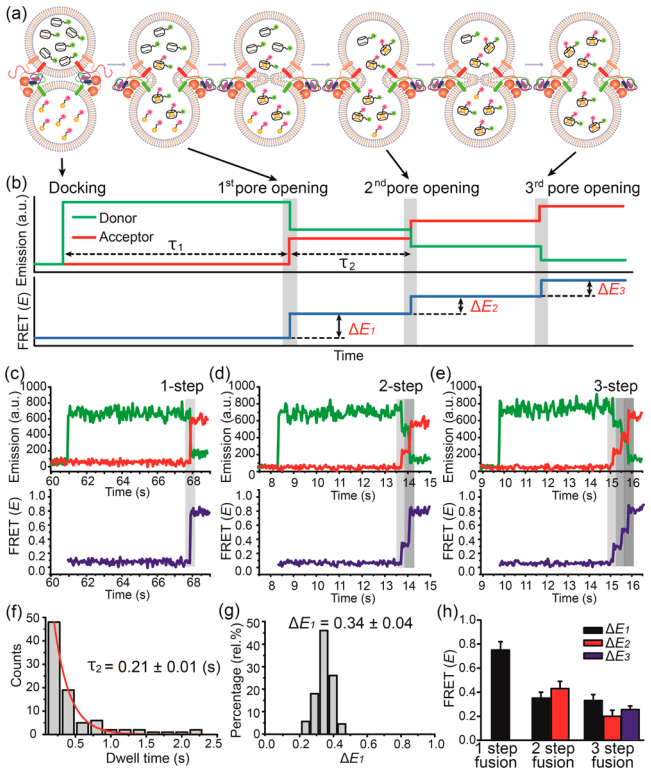

Figure 4. Observation of multistep fusion pore opening and closure dynamics. (a) Schematic illustration of fusion pore opening and closure events. (b) Expected time traces of Cy3 and Cy5 emission intensity during fusion pore flickering events. τ_1 denotes the dwell time between vesicle docking and the first fusion pore opening, while τ_2 denotes the dwell time of fusion pore closure state. (c–e) Representative fluorescence intensity time traces obtained by single-vesicle content mixing assay: (c) single-step fusion (72%), (d) two-step fusion (19%), and (e) three-step fusion (9% among 500 total time traces). (f) Distribution of τ_2, $\tau_2 = 0.21 \pm 0.01$ s. (g) Distribution of ΔE_1 of two- and three-step fusion cases. The mean of ΔE_1 is 0.34 ± 0.04. (h) Bar graph of average ΔE_1, ΔE_2, and ΔE_3 for multistep fusion cases.

FRET signal increment would be observed, which should accompany multistep anticorrelated intensity changes between CB[7]-Cy3 and Ad-Cy5 fluorescence (Figure 4b). In fact, when we analyzed the individual fusion events, we observed multistep FRET increments from the real time traces of single-vesicle pairs (Figure 4c–e). While in most (~72%) of the fusion events, the FRET signal increased sharply in a single step, in 28% of the cases, the signal increased in two (19%) or even three (9%) steps (Figure S20).

Considering that in our FRET assay, each vesicle contains on average 40 molecules, it is unlikely that these steps are an artifact caused by small fluctuations in the binding of one or two host–guest pair molecules. Furthermore, if the steps occurred specifically due to the sensor molecules (CB[7] and/or Ad), we would expect the frequency of steps to change with sensor molecule concentration. However, even when the experiment was repeated with a range of host/guest molecule concentrations (from 400 to 800 μM), similar distributions of multistep events were obtained (Figure S20). Another possible scenario of artifact is the binding of multiple *v*-vesicles on a single *t*-vesicle immobilized on the surface. The event of *v*-vesicle binding is directly monitored by the increase of I_D (Figure 4c–e): the binding of another *v*-vesicle would increase I_D or total intensity of Cy3 and Cy5 in the time trace. In our measurement condition, however, we did not observe such multiple binding events. Furthermore, we tested the clustering of *v*-vesicles by fluorescence correlation spectroscopy measurement to exclude the possibility that multiply-clustered *v*-vesicles induce the multiple FRET increase during content mixing. No clustering of *v*-vesicles was observed in our experimental conditions (Figure S22). Lastly, we tested whether our content molecules were stuck to lipid membrane during reconstitution. We incubated a mixture of the probes and vesicles overnight and found that binding of Ad-Cy5 or CB[7]-Cy3 to lipid membrane is negligible (Figure S13). Hence, the intermediate steps we observed appear to be a property of the content mixing itself. More specifically, the intermediate steps observed in the fusion traces are consistent with what would be expected if there were transient contractions and redilations of the pore (Figure 4a,b). To the best of our knowledge, this is the first time that such a flickering of the pore has been observed in an *in vitro* neuronal SNARE-mediated vesicle fusion.[4c,7b,8]

To understand the underlying details of fusion intermediates and flickering events, we analyzed the dwell time (τ_1) between vesicle docking and initial fusion pore formation (Figure 4b). The distribution of τ_1 shows single exponential decay with a time constant of $\tau_1 = 6.8 \pm 0.6$ s (Figure 3c). By contrast, the dwell time between the closure and reopening of the pore (τ_2) during fusion pore flickering (Figure 4b), only measurable in our FRET system, is considerably shorter (~0.20 s) than τ_1 (Figure 4f). Our result implies that the initial fusion pore formation event faces a larger energetic barrier than the subsequent flickering events and suggests the initial fusion pore opening is the most time-consuming step during the vesicle fusion processes.

An extra merit of our system is that we can measure the amount of content released during each fusion pore flickering step by analyzing ΔE, which represents the degree of content releasing. In case of multistep fusion events, we define ΔE_1, ΔE_2..., ΔE_n as the FRET increment of the first through *n*th pore-opening steps (Figure 4b). In two-step processes, ΔE_1 (0.34 ± 0.04) was slightly smaller than ΔE_2 (0.43 ± 0.06) (Figure 4d,h). In three-step fusion case, the FRET increment for the first step ($\Delta E_1 = 0.33 \pm 0.03$) was close to that for the two-step fusion case, whereas those for the second ($\Delta E_2 = 0.20 \pm 0.05$) and third ($\Delta E_3 = 0.25 \pm 0.03$) steps were similar to each other but smaller than that of the first step (Figure 4e,h). These results imply that the initial pore opening event is similar for both two-step and three-step fusion events. When the second pore opening is not big enough, the third pore opening seems to be required to release the contents completely.

The neuronal exocytosis studies at the cellular level reported that fusion pores can open and close quickly (flickering) during fusion process.[3,9b,14] Because of the short-lived nature and complexity involved in these events, however, their detailed study using well-defined reconstituted *in vitro* system is highly challenging. Even though content-mixing assays are reliable to

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study the fusion pore dynamics, the fusion pore flickering events have not been observed by existing techniques due to the intrinsic limitations.[4c,7b,15] For instance, since only a few DNA-hairpin molecules can be encapsulated per vesicle, DNA-hairpin method cannot effectively detect any intermediate steps. Moreover, the content-mixing kinetics of DNA hairpin was considerably slower than that of small cargoes such as SRB[7b] suggesting that the hairpin can only detect large pores (>∼4 nm). The self-quenching SRB molecules are comparable in size with CB[7] and Ad (hydrodynamic radius ∼0.82 nm for CB[7]-Cy3 and ∼0.79 nm for Ad-Cy5, Figure S6b and S8b). However, the SNR of SRB is not good enough to observe the intermediate steps. Compared to these methods, our single-vesicle content-mixing assay successfully recapitulates the fusion pore flickering events and measured the kinetics of intermediate steps involved in fusion process (Figure 4). The flickering events that we observe are highly reminiscent, at least qualitatively, of neuronal kiss-and-run events.[9b,14b,16]

In conclusion, we have presented a supramolecular based single-vesicle content-mixing assay using the novel host−guest FRET pair as cargo contents to study the challenges of SNARE-mediated membrane fusion. The small size of our content molecules allows us to encapsulate up to an average of 80 molecules per vesicle with relative ease, while the extremely high binding affinity, which is required for a single-molecule assay, and specificity make the host−guest pair an ideal platform for single-vesicle fusion assay. Our assay revealed that the neuronal SNARE-mediated fusion can undergo multiple opening-and-closure events. The results presented here demonstrate the potential of the CB[7]-Ad FRET pair for the development of other single-molecule level biochemical assays. Specifically, we envision that by changing the labeling dye molecules, multicolor FRET can be applied to observe both lipid mixing and content-mixing or even SNARE protein interactions and content-mixing simultaneously.[17] Such applications should enhance our ability to study short-lived events in biologically complex processes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental methods and figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05385.

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Notes

The authors declare no competing financial interest.

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