Chapter 4

Dynamic Light Scattering Analysis to Dissect Intermediates of SNARE-Mediated Membrane Fusion

Byoungjae Kong, Yoosoo Yang, and Dae-Hyuk Kweon

Abstract

Dynamic light scattering (DLS) spectroscopy provides rapid information on the size distribution of a large number of particles in a mixture. Vesicle sizes change during the merger of lipid bilayers, and DLS analysis can provide rapid, accurate, and non-perturbative quantification of the size distribution of proteoliposomes in SNARE-dependent membrane fusion. In this chapter, we describe the methodologies and reagents used for DLS spectroscopy in a biochemical and biophysical study of SNARE-mediated membrane fusion.

Key words Dynamic light scattering, SNARE, Membrane fusion, Size distribution

1 Introduction

Dynamic light scattering (DLS) measures the translational diffusion coefficients (Dt) of nanoparticles and colloids in solution by quantifying the fluctuations of scattered light over time. The sizes and size distributions are then calculated from the diffusion coefficients based on hydrodynamic radius (R_H) or hydrodynamic diameter (D_H) [1].

DLS is used to estimate populations or aggregates of proteins or liposomes, and to check the quality of biomolecules prior to other analyses [2]. In particular, DLS can determine the size distribution of spherical nanoparticles such as vesicles without fractionation, and provides specific information on hydrodynamic radii and polydispersity. Based on the premise that vesicles are spherical, DLS measurements of polydisperse vesicles are usually analyzed to determine moments of their size distributions, average vesicle size, and polydispersity (relative variance) [3, 4].

Here we introduce the DLS technique as an analytical tool for the study of SNARE-mediated membrane fusion [5]. The SNARE

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proteins are in a large protein superfamily, consisting of target SNAREs (T-SNAREs) and vesicle SNAREs (V-SNAREs), which mediate all intracellular membrane fusion events. For example, in a neuron, a T-SNARE (syntaxin 1a and SNAP-25) on the presynaptic plasma membrane and a V-SNARE (synaptobrevin) on the synaptic vesicle [6–8], which contains neurotransmitter, bridge the two membranes in the *trans* conformation to trigger fusion [9–12].

SNARE-mediated membrane fusion proceeds via hemifusion intermediates before opening of a fusion pore [13–15]. Hemifusion, in which the outer leaflets of membranes are fused, but the inner leaflets remain separate (without content mixing), begins with formation of a hemifusion stalk. The hemifusion stalk is an hourglass-shaped lipid structure that has a local connection with the outer leaflets, and expansion of the stalk leads to formation of the hemifusion diaphragm. A fusion pore may form from the hemifusion diaphragm or directly from the stalk [16]. There is evidence that Ca^{2+} may trigger rapid transition to full fusion from the point of contact [17].

Three methods allow observation of the hemifusion state during membrane fusion. First, the hemifusion state can be monitored using bulk fluorescence-based assays, such as *the SNARE-driven total lipid mixing assay* [8] and *the inner leaflet mixing assay* [15, 18]. To determine the proportion of hemifused vesicles during a fusion reaction by *the inner leaflet mixing assay* [15], fluorescent V-vesicles are pretreated with 2.5 mM sodium dithionite, which selectively inactivates NBD-PS fluorescent dyes in the outer leaflets. Subsequently, the percentage of hemifused vesicles can be calculated as a function of time: $2(P_T - P_I)/[(2(P_T - P_I) + P_I)] \times 100\%$, where P_T is the total lipid mixing percentage and P_I is the inner lipid mixing percentage (Fig. 1a) [8, 14, 19–23].

Second, the fluorescence resonance energy transfer (FRET)-based lipid and content mixing assay provides information at the level of single vesicles, and allows identification of the different stages of fusion [24]. In particular, this method simultaneously monitors content and lipid exchange, and can differentiate single-vesicle interaction, hemifusion, and complete fusion. Complete fusion mimics quantized neurotransmitter release upon exocytosis of synaptic vesicles (Fig. 1b).

Third, cryo-electron microscopy (EM) can provide ultrastructural information of the liposomes in which there is a single bilayer or double bilayers between the two lumens; however, this method cannot easily distinguish small differences in the counts of docking and hemifusion (Fig. 1c) [25].

Compared to methods that use FRET or cryo-EM, DLS provides precise information concerning the progression of membrane fusion, including the composition of all fusion intermediates, in which data are averaged over a large number of particles within a short period. In addition, DLS measurements using a conventional

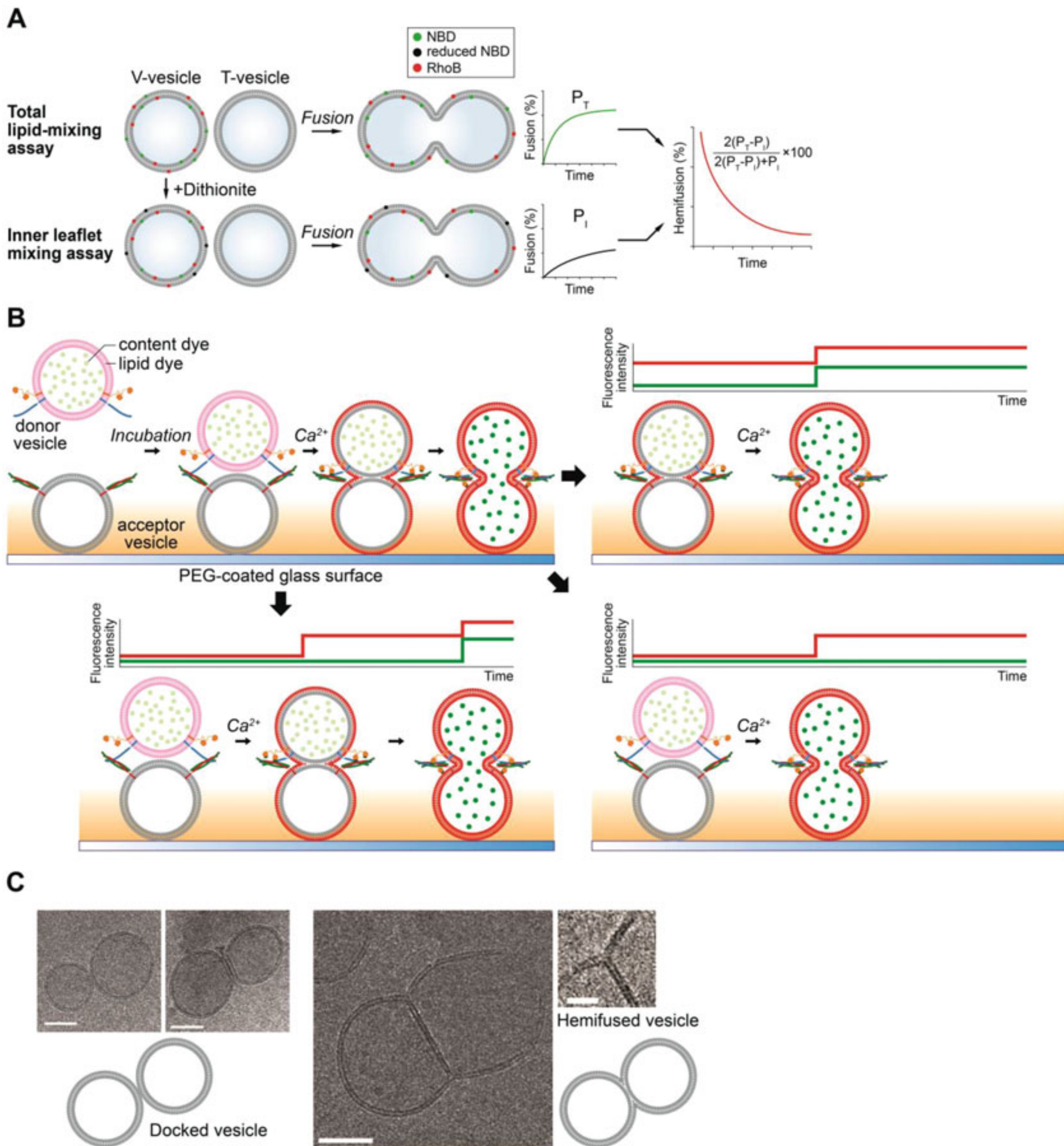


Fig. 1 Methods used to observe hemifused vesicles in SNARE-mediated membrane fusion. (**a**) Fluorescence-based assays (SNARE-driven total lipid mixing assay and inner leaflet mixing assay). For the total lipid mixing assay, V-vesicles labeled with 1,2-dioleoyl-*sn*-glycero-3-phosphoserine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PS) (green) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE) (red) are mixed with non-fluorescence-labeled T-vesicles, leading to decreased fluorescence quenching and increased signal from the donor (NBD-PS). For the inner leaflet mixing assay, dithionite is added to the samples before fusion to completely quench NBD (black); the NBD of the inner leaflet remains protected from dithionite. The percentage of hemifused vesicles is obtained by $2(P_T - P_I)/[(2(P_T - P_I) + P_I)] \times 100\%$, where P_T is the total lipid mixing percentage and PI is the inner lipid mixing percentage. (**b**) Single-vesicle content and lipid mixing system. Donor vesicles labeled with fluorescent indicator dye (light green) and fluorescent lipid dye (pink) are added to acceptor vesicles tethered to a PEG-coated glass surface. After incubation to allow fusion and injection of a Ca^{2+} solution, the fluorescence signal is measured in two channels, along with the dilution of the two self-quenching fluorescent dyes. (**c**) Ultrastructural cryoEM images of vesicle docking and hemifusion [25]. Reprinted with permission from American Association for the Advancement of Science

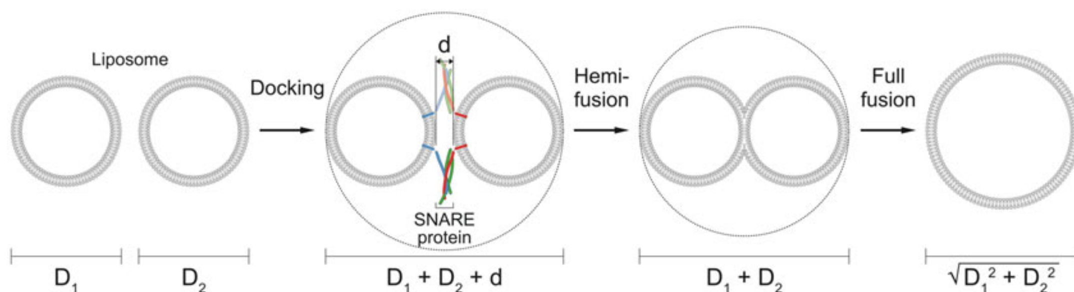


Fig. 2 Size changes during progression of membrane fusion. Full fusion of liposomes (initial diameters: D_1 and D_2) leads to a diameter of $(D_1^2 + D_2^2)^{1/2}$, whereas hemifusion leads to a diameter of $D_1 + D_2$. Reprinted with permission from Elsevier

cuvette-based system require relatively small sample volumes. DLS can rapidly, accurately, and noninvasively track and quantify the size distribution of proteoliposomes in SNARE-mediated membrane fusion processes. In particular, this in situ dye-free analysis can be completed in less than 200 s [2, 26, 27]. This method measures the changes in scattered light intensity over time, based on the autocorrelation of the intensity trace. The autocorrelation decay is then used to determine the molecular diffusion coefficient, from which the hydrodynamic radius is calculated.

The fusion intermediates can be distinguished by their unique average hydrodynamic radii. During SNARE-mediated membrane fusion, the size of vesicles varies according to the fusion step. Thus, docked vesicles, which have a gyration diameter of $2D + d$ (D, diameter of each vesicle; d, intermembrane distance), are formed by incubation of T- and V-vesicles at 4 °C; and it is possible to split the docked vesicles by digestion of SNARE proteins with proteinase K (PK). When two vesicles have fully merged, their hydrodynamic diameter can be calculated as $\sqrt{2}D$. A diameter of $2D$ indicates progression from vesicle docking to hemifusion (Fig. 2).

DLS can be used as a semiquantitative pre-screening method to rapidly identify part of the process of SNARE-mediated membrane fusion. Given that the DLS instrument is easy to use and detailed knowledge of the underlying physics of molecular sizing is not needed, we only provide a brief explanation here.

2 Materials

2.1 Instrument Preparation

1. DynaPro® DLS instrument (Wyatt Technology, Goleta, CA, USA).
2. Instrument software: DYNAMICS (version 6.0) installed on a PC.
3. 45 μL quartz cuvette (Wyatt Technology).

4. Water for cleaning cuvette.
5. Lens cleaning paper.
6. 10–20 μL Sample.
7. Compressed air.
8. Dialysis buffer: 25 mM HEPES, 100 mM KCl, pH 7.4.

2.2 Sample Preparation

2.2.1 Expression and Purification of SNARE Proteins

1. Incubator/shaker set at 37 °C for cell culture.
2. −80 °C Lab freezer for long-term storage.
3. Refrigerated ultracentrifuge and appropriate tubes for cell harvesting.
4. Spectrophotometer.
5. Probe-type ultrasonicator.
6. *Escherichia coli* CodonPlusRIL (DE3) cells containing pGEX-2T-1 vector encoding a thrombin-cleavable N-terminal glutathione S-transferase tag to purify SNARE proteins: full-length syntaxin 1a (amino acids 1–288), full-length VAMP2 (amino acids 1–116), and SNAP-25 (amino acids 1–206) (*see* **Note 1**).
7. 25 g/L Luria-Bertani (LB) broth (Miller) in 2 L baffled flask: Sterilized by autoclaving.
8. Gravity-flow purification column.
9. Glutathione-agarose bead equilibrated with PBS (*see* Subheading 2.2.1, **item 18** and **Note 2**).
10. 50 mg/mL Ampicillin in H_2O: Sterilized by filtration and stored at −20 °C.
11. 1 M Isopropyl β-D-thiogalactoside (IPTG) in H_2O for induction: Sterilized by filtration and stored at −20 °C.
12. 0.2 M 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) in H_2O as protease inhibitor: Sterilized by filtration and stored at −20 °C.
13. 1 M Dithiothreitol (DTT) in H_2O: Sterilized by filtration and stored at −20 °C.
14. 0.5 M Ethylenediaminetetraacetic acid (EDTA) in H_2O: Sterilized by filtration.
15. 10% *n*-Octyl-D-glucopyranoside: Sterilized by filtration.
16. 1000 U/mL Thrombin for elution.
17. Glycerol as a cryoprotectant: Sterilized by autoclaving.
18. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4.
19. PBST buffer: 0.05% Tween 20, 0.1% TritonX-100 in PBS, pH 7.4.
20. Thrombin cleavage buffer (TCB): 50 mM Tris–HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.0.

2.2.2 Preparation of Unilamellar Liposomes

1. Laboratory fume hood.
2. Vacuum desiccator with air pump.
3. Round-bottom glass test tube.
4. Nitrogen gas.
5. Phospholipids (Avanti Polar Lipids Inc., Alabaster, AL, USA): 1-Palmitoyl-2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PS), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE).
6. Laboratory water bath set to 37 °C.
7. Gastight syringes.
8. Polycarbonate membrane with 100 nm pores.
9. Filter supports.
10. Extrusion equipment: Refer to https://avantilipids.com/divisions/equipment for details.
11. PBS (*see* Subheading 2.2.1, **item 18**).

2.2.3 Reconstitution of SNARE Proteins Into Membranes

1. Magnetic stirrer.
2. 1 L Glass beaker.
3. Dialysis unit (10,000 molecular weight (MW) cutoff).
4. 10% *n*-Octyl-D-glucopyranoside: Sterilized by filtration.
5. Aluminum foil.
6. Bio-Beads SM-2 polystyrene adsorbent (Bio-Rad, Hercules, CA, USA): Wash with methanol, thoroughly rinse with water, and then store in water. Amberlite XAD-2 beads (Sigma catalog number 20275 or 10357) can also be used.
7. Dialysis buffer (*see* Subheading 2.1, **item 8**).

2.3 DLS Assay

1. Polyphenolic compounds (Sigma-Aldrich) dissolved in DMSO.
2. 15 mg/mL Proteinase K (PK) stock solution (100×): Stored in aliquots at −20 °C.
3. 0.1 M Phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor.

3 Methods

Perform all procedures in the order given below. Begin with sample preparation, such as reconstitution of purified proteins into liposomal membranes; then perform DLS measurements, during the membrane fusion processes; and finally, analyze and interpret the DLS results.

3.1 Preparation of Instrument

3.1.1 Measuring DLS Data

Before membrane fusion experiments, perform multiple DLS measurements to confirm the proper operation of the instrument, as described in the technical documentation supplied by Wyatt (http://www.wyatt.com).

1. First, turn on the DynaPro® DLS instrument and the connected PC, and then start the DYNAMICS software.
2. In the software, open a "New" experiment, and then click the "Connect" button on the upper left side of the Experiment window to connect the instrument and software. Ensure that the color of the "Laser" tab in the instrument display changes from yellow to green.
3. Set the variables, including temperature, using the Parameters—Instrument node.
4. Place the quartz cuvette containing a sample in the sample holder on the optics block (*see* **Note 3**). The angled corner of the cuvette must point to the angled corner of the holder, as marked on the instrument. Before starting DLS measurements, make sure that all the variation tabs on the instrument board have changed from yellow to green.
5. Click the green "Start" button in the Experiment window toolbar to initiate DLS measurements.
6. To export the data, right-click, click "Export," and save as an Excel file.
7. After completion of experiments, save the software file, close the software, and turn off the instrument and the PC.

3.1.2 Cuvette Cleaning

Thoroughly clean the DLS cuvettes and other devices, because dust and other large particles can disturb DLS signals. More specifically, clean the cuvette before and after use, do not scratch the cuvette during cleaning, and thoroughly clean all dust from the outer surface of the cuvette. Perform cleaning as follows (*see* **Notes 4** and **5**).

1. First, perform multiple washes of the interior of the cuvette with a 1% TritonX-100 solution.
2. Rinse the cuvette three to five times with deionized water.
3. Dry the interior of the cuvette using compressed air.
4. Wipe the outer surface of the cuvette with lens-cleaning paper to remove dust, being careful not to scratch the exterior. Also, make sure that there is no dust on the cap of the cuvette.

3.2 Preparation of Sample

3.2.1 Expression and Purification of Recombinant SNARE Proteins

First, test the overexpression of SNARE proteins in *E. coli* grown in a small-scale culture to identify the best conditions (e.g., optimal strain and temperature of induction for soluble expression). Follow the previously described procedures of protein expression and purification for neuronal SNARE proteins [28]. SNARE proteins can be expressed using the pGEX expression system with CodonPlus-RIL(DE3) strain as a host. Perform this procedure as follows:

1. Prepare a starting culture by adding 10 mL of LB medium into a glass culture tube containing 50 μg/mL ampicillin that was inoculated (0.1% [v/v]) with a cell culture stock from single colony. Grow the culture at 37 °C with shaking (250 rpm) overnight.
2. Autoclave 600 mL of LB medium in a 2 L baffled flask supplemented with 50 μg/mL ampicillin that was inoculated with 1% (v/v) of overnight-grown culture and subsequently incubated at 37 °C at 150 rpm. When the OD_{600} reaches 0.6–0.8 (usually in 2–3 h), perform induction with 0.5 mM of IPTG. For the highest yield, the post-induction time, temperature, and shaking speed of the cell culture are 6–12 h, 16 °C, and 100 rpm for syntaxin 1a, 4 h, 26 °C, and 100 rpm for SNAP-25, and 6 h, 22 °C, and 100 rpm for VAMP2, respectively (*see* **Note 6**).
3. Harvest cells by centrifugation at 6000 × *g* for 10 min, and store the pellet at −80 °C.
4. Resuspend the cell pellet in 10 mL of chilled buffer (PBS for soluble SNAP-25 and PBST for insoluble syntaxin 1a and VAMP2), and then lyse the cells by sonication (45% amplitude, 1.5 min net sonication, 1 s on/1 s off) in the presence of 1 mM AEBSF (*see* **Note 7**).
5. Clarify the lysate by centrifugation at 13,000 × *g* for at least 20 min, and then perform binding to 2 mL of glutathione-agarose beads equilibrated with PBS or PBST for 2 h at 4 °C with constant agitation (*see* **Note 2**).
6. Wash the column:
   (a) First with 10-column volumes (300 mL) of PBS or PBST
   (b) Then with 5 mL of TCB (for soluble proteins) or TCB supplemented with 1% *n*-octyl-D-glucopyranoside (for insoluble proteins)
7. Elute SNARE proteins by adding 20 U of thrombin to the column, followed by overnight incubation at 4 °C to allow thrombin digestion at cleavage sites on the vector. Monitor the progress of the cleavage reaction by SDS-PAGE.
8. Store the eluted SNARE proteins at −80 °C with 1 mM AEBSF and 10% (v/v) glycerol. Determine the protein concentration using the Bradford assay with bovine serum albumin as the standard, and confirm protein purity using SDS-PAGE.

3.2.2 Preparation of Unilamellar Liposomes

Prepare large unilamellar vesicles of various diameters by extrusion through polycarbonate filters from Avanti Polar Lipids. For the bulk lipid mixing assay, use a molar ratio of POPC to DOPS for T-vesicles of 65:35, and for V-vesicles a POPC:DOPS:NBD-PS: rhodamine-PE molar ratio of 62:35:1.5:1.5.

1. Prepare lipid stocks in chloroform, and store at −20 °C in glass vials. Prior to use, maintain the lipids at 25 °C for at least 30 min.
2. Dispense the desired amount of lipid stock into a disposable glass tube, and dry it using a gentle stream of nitrogen gas in a fume hood to remove solvents.
3. Place the resultant thin lipid film on the lower walls of the tube in a vacuum desiccator under high vacuum overnight (>2 h) to eliminate residual solvents.
4. Add dialysis buffer to the dried lipid film, and then vortex the tube for hydration (*see* **Note 8**).
5. Subject the lipid solution to 5 freeze-thaw cycles, followed by an extrusion step with 21 passes through a polycarbonate membrane (100 nm pores) (*see* **Note 9**).
6. Store the extruded liposome samples at 4 °C until use.

3.2.3 Reconstitution of SNARE Proteins Into Membranes

Reconstitute the binary T-SNARE complex into T-vesicles and VAMP2 into V-vesicles, as described elsewhere [23, 28, 29]. Perform octyl glucoside-assisted insertion [28] of the T-SNARE complex (a binary complex of syntaxin 1a and SNAP-25) or VAMP2 to form populations with effective monodisperse size distributions. Perform this procedure as follows:

1. Mix the purified syntaxin 1a and SNAP-25 at room temperature for 1 h to allow formation of the T-SNARE binary complex. Use additional *n*-octyl-D-glucopyranoside to keep the detergent concentration in the syntaxin 1a above the critical micelle concentration (CMC), which is 0.025 M (~0.7% w/v) for *n*-octyl-D-glucopyranoside (*see* **Note 10**).
2. Mix the T-SNARE complex with the prepared unilamellar vesicles at a lipid:protein molar ratio of 50:1. For the V-SNARE vesicles, mix 10 mM fluorescence-labeled liposomes with VAMP2 at a lipid:protein molar ratio of 50:1, and then incubate at 25 °C for 20 min. Subsequently, dilute the liposome/protein mixture twofold using dialysis buffer, so that the concentration of *n*-octyl-D-glucopyranoside is below the CMC.
3. Dialyze the samples against dialysis buffer using the dialysis unit (MW cutoff 10,000) at 4 °C overnight to remove the detergent. Provide constant agitation during dialysis for efficient removal of detergent by stirring the dialysis unit inserted into the floating tube rack in a large beaker. Wrap the beaker with aluminum foil to protect the contents from light.

4. After dialysis, transfer the samples to new tubes, add the Bio-Beads SM-2 adsorbents to eliminate any remaining detergent, and then incubate at 4 °C for 30 min.
5. Centrifuge the sample at 13,000 × *g* for 30 min to remove protein/lipid aggregates and then determine the reconstitution efficiency using SDS-PAGE. Estimate the amount of protein in the liposomes using densitometry to compare the band in the gel with a known concentration of the same protein.

3.3 SNARE-Driven Membrane Fusion Using DLS

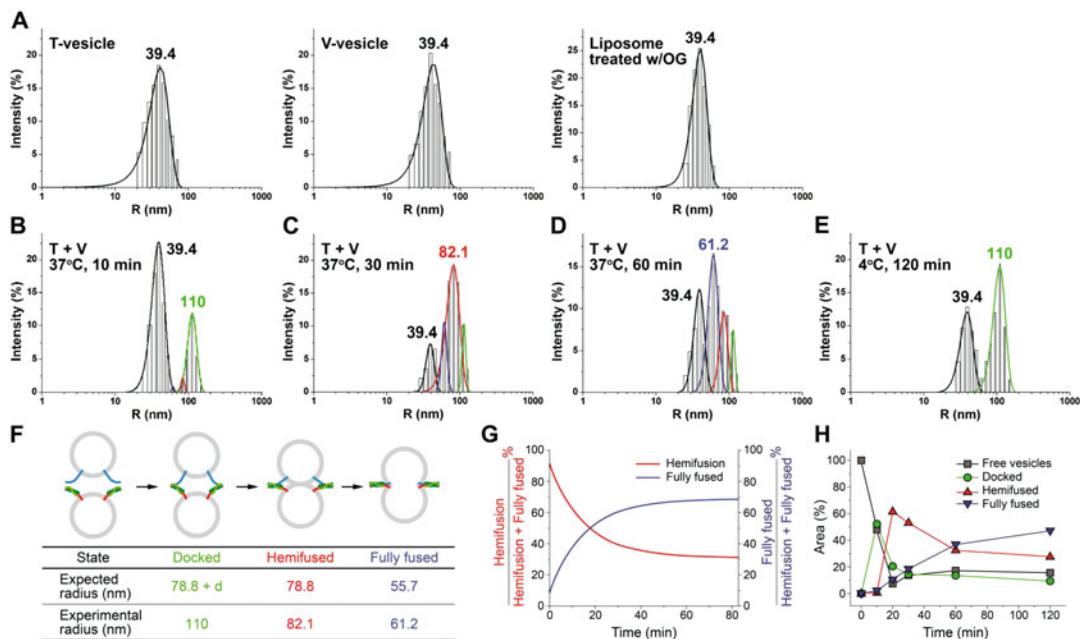
1. Mix the T- and V-vesicles in a 1:1 ratio, so that the reaction mixture contains 0.5 mM each. Optional: Add SNARE inhibitors (e.g., small polyphenols) to one vesicle to inhibit SNARE complex formation, and then mix with the other vesicle for initiation of fusion (*see* **Note 11**).
2. Incubate the sample at 37 °C to allow fusion (0–120 min), and then dilute the sample 100-fold using dialysis buffer so that the final lipid concentration is 10 μM. Incubate another set of the samples at 4 °C, a condition that only allows vesicle docking, not lipid mixing.
3. Optional: Directly add the 100× proteinase K (PK) stock solution to the sample, and incubate at 4 °C or 37 °C for 1 h. Terminate the reaction by adding the protease inhibitor (0.1 M PMSF). Determine the connection of the outer leaflets of bilayers using PK, which digests SNARE proteins and allows discrimination between hemifused and docked vesicles.
4. Centrifuge the sample at 13,000 × *g* at 4 °C for 5 min, and collect the supernatant for DLS measurements (*see* Subheading 3.1 and **Note 12**).
5. Use the DYNAMICS software to calculate the radii and the size distributions based on the regularization algorithm and interpret them (*see* **Notes 13** and **14**).

3.4 DLS Data

3.4.1 Hydrodynamic Diameters of Fusion Intermediates

When two vesicles fuse, the outer leaflets of the bilayers merge, resulting in a hemifused vesicle, in which the inner leaflets remain separate, yielding a peanut-like structure. If the diameters of the opposing membranes are D_1 and D_2, then the diameter of the hemifused vesicle is simply $D_1 + D_2$. The gyration of this peanut-like structure in solution will appear as a large sphere (Fig. 2). During inner leaflet mixing, the hemifused membrane becomes a larger single membrane. Thus, the size of the full fusion product is $(D_1^2 + D_2^2)^{1/2}$ because the surface area of a full fusion product is the sum of the surface areas of the two merging vesicles (Fig. 2). The hydrodynamic diameter of the docked vesicles is $D_1 + D_2 + d$, where d is the intermembrane distance (*see* Example 1).

Monodisperse proteoliposomes consisting of T-vesicles (containing T-SNARE proteins) and V-vesicles (containing V-SNARE VAMP2) that were prepared by reconstitution methods had mean



Example 1 Dynamic light scattering (DLS) assay of membrane fusion. (**a**) DLS analysis of liposomes prepared by extrusion and treatment with octyl glucoside. (**b–d**) T- and V-vesicles were mixed and incubated at 37 °C for 2 h, and samples were analyzed by DLS at 10, 30, and 60 min. A sum of four Gaussian distributions were used to fit the DLS histograms after setting the centers at 39.4, 61.2, 82.1, and 110 nm. (**e**) T- and V-vesicles were mixed and incubated at 4 °C, leading to formation of docked vesicles that had hydrodynamic radii of 110 nm. (**f**) Comparison of the expected and experimental values of radii of each fusion intermediate when they evolved from T- and V-vesicles with radii of 39.4 nm. (**g**) Percentage of hemifusion was calculated from sodium dithionite-treated V-vesicles. (**h**) Time-dependent traces of each fusion intermediate. All DLS histograms (**b–d**) obtained during the 2-h fusion reactions were decomposed by means of a fourfold Gaussian fit into each component representing fusion intermediates. The centers of these Gaussians were 39.4, 61.2, 82.1, and 110 nm, and the area under each was used to indicate the fraction of free vesicles, fully fused vesicles, hemifused vesicles, and docked vesicles, respectively. The FRET and DLS assays both indicate a time-dependent increase in the number of fully fused vesicles, and a decrease in the number of hemifused vesicles. This confirms that DLS quantitatively identifies all fusion intermediates. All materials reprinted with permission from Elsevier

hydrodynamic radii of 39.4 nm (Example 1a). The size of unreacted free vesicles (radius: 39.4 nm) remained unchanged, and was clearly different from that of other fusion intermediates. After mixing the two vesicles, four major peaks appeared (mean radii of 39.4, 61.2, 82.1, and 110 nm), depending on the incubation time (Example 1b–d). Docked vesicles, formed following incubation of T- and V-vesicles at 4°C for 2 h, with an expected gyration diameter of $D_1 + D_2 + d$, have hydrodynamic radii of 110 nm (Example 1e). The value 82.1 nm is similar to the sum of two vesicles, each with a radius of 39.4 nm (78.8 nm; expected hydrodynamic radius of hemifused vesicles). The fully fused vesicles had measured radii of 61.2 nm, similar to the value from the above

equations (55.7 nm) (Example 1f). When T- and V-vesicles were mixed and the fusion reaction proceeded at 37°C, vesicles with hydrodynamic radii of 61.2 and 82.1 nm appeared. Over time, the fraction of 61.2 nm vesicles increased and the proportion of 82.1 nm vesicles decreased (Example 1g, h).

3.4.2 Discrimination of Hemifused Vesicle Pairs from Docked Vesicle Pairs Using PK

The hallmark of hemifusion is a connection between the outer leaflets of bilayers. A docked vesicle pair is linked via partially zipped SNARE proteins; a hemifused vesicle pair has two membranes connected by the continuum of the outer leaflets. Thus, a docked vesicle pair should split into separate vesicles following digestion of the SNARE proteins with PK, whereas a hemifused vesicle pair should be resistant to PK (*see* Example 2).

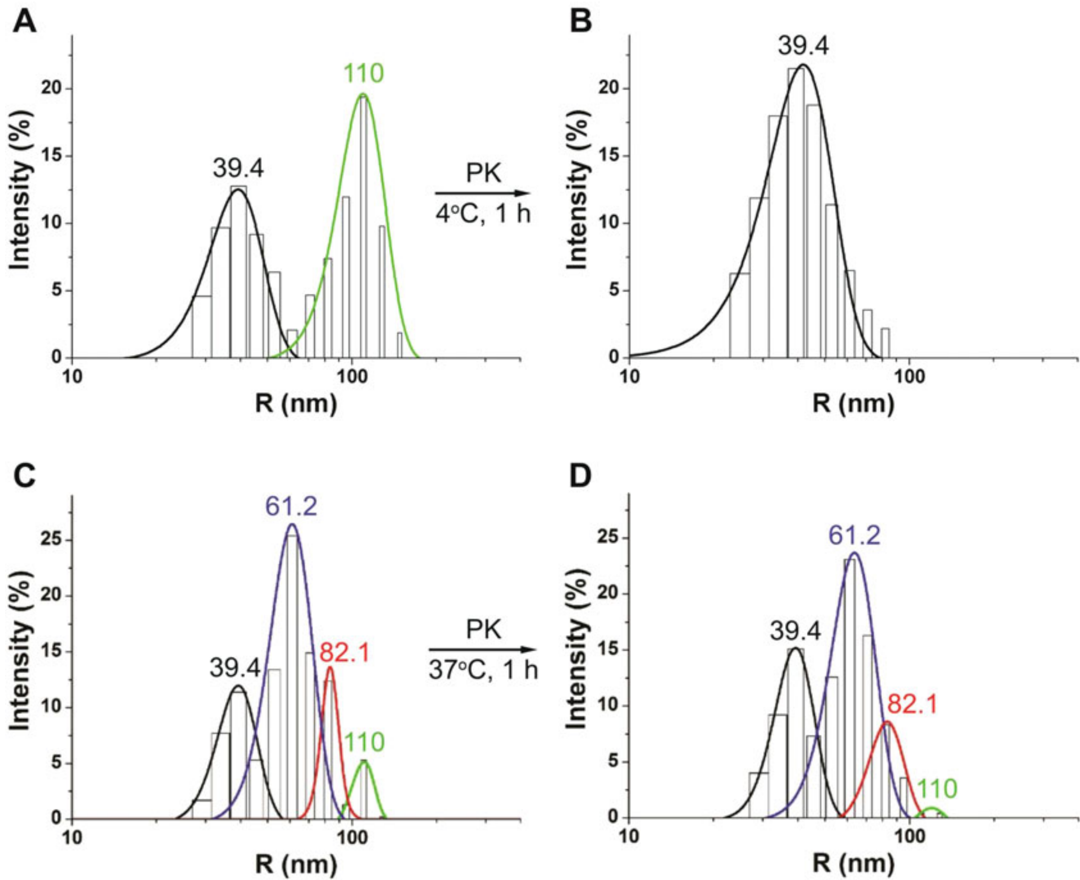
1. Docked vesicles: T- and V-vesicles were mixed at 4°C, incubated for 2 h, and treated with PK for 60 min. A 110 nm peak disappeared after PK treatment (Example 2a, b), confirming that the DLS peak at a radius of 110 nm corresponded to the docked vesicle pairs.
2. Fused vesicles: T- and V-vesicles that contain the docked vesicle pairs (enriched by preincubation at 4 °C) were incubated at 37 °C for 1 h, leading to a decline in the 110 nm peak, and increased full fusion and hemifusion peaks (Example 2c). Treatment of these vesicles with PK for 1 h led to the disappearance of the 110 nm peak, and a slight increase in the free vesicle peak (Example 2d).

3.4.3 The DLS Assay of SNARE Complex Inhibitors

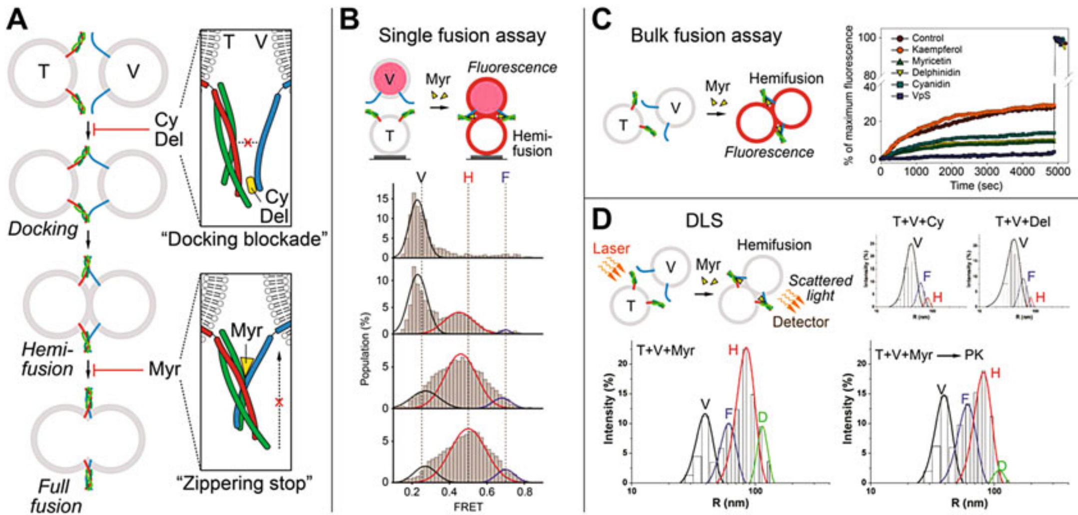
The DLS-based fusion assay was used to analyze the mode of action of several blockades that inhibit SNARE-mediated membrane fusion. DLS can be used to determine whether the inhibition of SNARE-mediated membrane fusion by several flavonoids corresponds to the phenomena previously identified by FRET (*see* Example 3).

We recently showed that several flavonoids inhibit formation of the SNARE complex, thereby inhibiting membrane fusion. More specifically, delphinidin and cyanidin interfere with vesicle docking by binding to the N-terminus of the SNARE complex; myricetin arrests membrane fusion in the hemifused state by wedging into the middle region of the core complex and halting SNARE complex formation midway through the zippering [28, 30] (Example 3a–c).

1. Delphinidin and cyanidin prevent docking of vesicles. These inhibitors suppress docking, such that the vesicles have no interactions.
2. Myricetin arrests membrane fusion in the hemifused state (Example 3d). This inhibitor leads to enrichment of vesicles with hydrodynamic radii of 82.1 nm during SNARE-driven membrane fusion. These hemifused vesicles were unaffected by PK treatment. This is consistent with the results of the bulk and single FRET assays of membrane fusion [28, 30].



Example 2 Use of DLS to discriminate docked vesicles from other fusion intermediates. (**a**) Mixture and incubation of T- and V-vesicles at 4°C for 1 h. (**b**) Treatment of the reaction mixture from (**a**) with PK at 4°C for 1 h led to complete disappearance of vesicles that had hydrodynamic radii of 110 nm, suggesting that they formed docked vesicles. (**c**) Incubation of T- and V-vesicles at 37 °C for 1 h led to formation of vesicles with hydrodynamic radii of 61.2 and 82.1 nm, corresponding to fully fused and hemifused vesicles, respectively. (**d**) Treatment of the reaction mixture from (**c**) with PK at 37 °C for 1 h led to splitting of docked vesicles (110 nm), confirming that vesicles that had hydrodynamic radii of 82.1 nm were connected by lipids. All materials reprinted with permission from Elsevier



Example 3 Comparison of DLS with fluorescence-based fusion assays. (**a**) Inhibition of membrane fusion by flavonoids. Delphinidin and cyanidin bind to the N-terminus of the SNARE complex, inhibiting vesicle docking; myricetin halts membrane fusion in the hemifused state by functioning as a "wedge" in the middle of the SNARE core complex. (**b**–**d**) Schematic of vesicle fusion state by a single-vesicle fusion assay [30] (**b**), conventional standard bulk fusion assay (**c**), and DLS (**d**). (**b**) Reprinted with permission from American Chemical Society. (**d**) Reprinted with permission from Elsevier

4 Notes

1. *Escherichia coli* CodonPlusRIL (DE3) strains are engineered to contain extra genes encoding the tRNAs that frequently limit translation of heterologous proteins in *E. coli*, such that they enable to express heterologous proteins at high level.
2. When glutathione agarose is provided in powder form, swelling of the lyophilized powder using water (200 mL/g) needs to be required. Generally, 90–95% of swelling occurs within 30 min at room temperature, but we prefer to recommend the overnight incubation at 4°C for 100% swelling. After swelling, wash the agarose beads thoroughly with water (10 volumes) or preferred equilibration buffer (e.g., PBS) to remove the lactose present in the lyophilized product. The resin can be stored in 2 M NaCl at 4 °C.
3. To avoid the formation of air bubbles, load the samples carefully with the pipette tip touching the bottom of the cuvette.
4. For efficient cleaning of the cuvette, it is recommended to use a dedicated cleaning washer.
5. Because dust particles in your sample can scatter light and affect the DLS analysis, use only filtered buffer in the cuvette and then fulfill a standard check to assure sufficient cleaning. Well-characterized proteins like BSA or ovalbumin as standard

samples to ensure that the DLS instrument works well can be used. Moreover, several standards such as 100 nm polystyrene latex spheres can be used to ensure function of the DLS instrument.

6. We have confirmed that culturing *E. coli* with temperature and shaking speed lowered is required for producing high yield of SNARE proteins. It is postulated that lower temperatures, or general slower growth conditions, increase the time that proteins have to fold, although this is not always borne out.
7. Usually, we disrupt and lyse the bacterial cells in a 50 mL conical tube using a 750 W ultrasonic processor with a probe at 45% amplitude. The cells are chilled on ice between pulses.
8. Lipid stocks are prepared in chloroform at 25 mg/mL and stored at −20 °C in glass vials. We routinely prepare unlabeled liposomes (without NBD-PS and rhodamine-PE) with a lipid concentration of 50 mM and fluorescently labeled liposomes with a 10 mM lipid concentration. For unlabeled liposomes (50 mM lipid), for example, this is achieved by hydration of a lipid film made of 98.9 μL of 25 mg/mL POPC (65 mol%) and 56.7 μL of 25 mg/mL DOPS (35 mol%) using 100 μL of dialysis buffer. At this point, careful and sufficient vortexing is required because the volume for hydration is generally small. Moreover, an ultrasonic bath can be used for completely dissolving the lipid film.
9. Lipid solutions were subjected to ten rapid cycles of freeze-thawing by submersion in liquid nitrogen and 42 °C water, respectively, for change of liposomal structure from multilamellar to unilamellar. Two gastight syringes and extruders for extrusion were thoroughly cleaned with 100% ethanol ten times before use. During the extrusion process, it is important to tightly assemble the extruder parts (extruder outer casing, nut, internal membrane supports, etc.) to prevent sample leakage. After the final pass, samples were collected in a clean microcentrifuge tube and stored at 4 °C until use.
10. Binary T-SNARE complex is prepared with syntaxin 1a and SNAP-25 at a ratio of 1:1, supplemented with 10% *n*-octyl-D-glucopyranoside, where the order of addition for the binary complex mixture is syntaxin 1a, 10% *n*-octyl-D-glucopyranoside, and SNAP-25 to maintain the CMC of *n*-octyl-D-glucopyranoside in a syntaxin 1a protein sample. Before the dilution step, the CMC of the detergent of the protein sample should be maintained above 0.8%.
11. We recommend to perform a lipid mixing assay in combination with DLS analysis for SNARE-mediated membrane fusion to confirm the functionality of the prepared proteoliposomes.

12. It is important that samples for DLS analysis should be clarified by various means (e.g., centrifugation or filtration) to achieve a high level of purity. Usually, before DLS measurements, the polished samples should be accurately re-quantified. At least ten acquisitions should be done for each sample and the measurements should be taken in triplicate for checking reproducibility.
13. DLS measurement is known to be very sensitive to temperature critical for analysis of SNARE-mediated membrane. Thus, the temperature must be strictly controlled and kept constant for a reliable DLS result.
14. The scattering intensity from DLS depends on the molecular size and concentration of the test samples, so the optimization of concentration should be required to obtain reliable measurements.

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