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## Size of Supramolecular SNARE Complex: Membrane-Directed Self-Assembly

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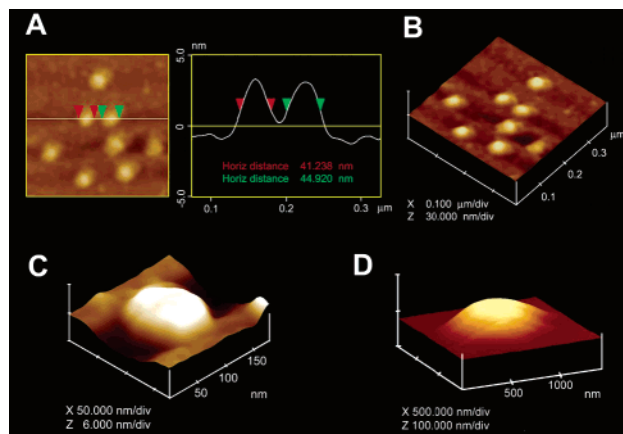
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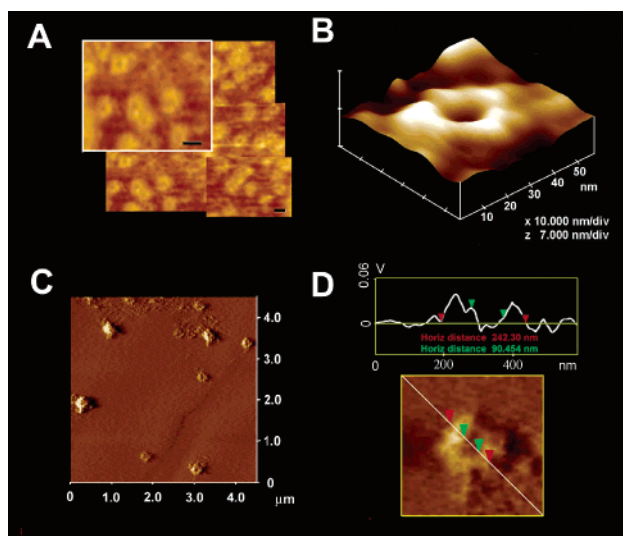
All life processes are governed at the chemical level. Knowledge of how single molecules interact provides a fundamental understanding of nature. An aspect of molecular interactions is the self-assembly of supramolecular structures. For example, membrane fusion requires the assembly of a supramolecular complex formed when proteins in opposing bilayers interact. Membrane fusion is essential for numerous cellular processes, including hormone secretion, enzyme release, or neurotransmission. In living cells, membrane fusion is mediated via a specialized set of proteins present in opposing bilayers.<sup>1,2</sup> Target membrane proteins, SNAP-25 and syntaxin (t-SNAREs) and secretory vesicle-associated protein (v-SNARE), are part of the conserved protein complex involved in fusion of opposing lipid membranes.<sup>1,2</sup> The structure and arrangement of the membrane-associated full-length SNARE complex was first examined using atomic force microscopy (AFM).<sup>3</sup> Results from the study demonstrate that t-SNAREs and v-SNARE, when present in opposing bilayers, interact in a circular array to form supramolecular ring complexes each measuring a few nanometers.<sup>3</sup> The ring complex helps in establishing continuity between the opposing bilayers.<sup>3</sup> In contrast in the absence of membrane, soluble v- and t-SNAREs fail to assemble in any specific pattern, or form such conducting pore structures.<sup>3</sup>

SNARE-ring complexes ranging in size from approximately 15 to 300 nm in diameter are formed when t-SNARE-reconstituted and v-SNARE-reconstituted lipid vesicles meet. Since vesicle curvature would dictate the contact area between opposing vesicles, this broad spectrum of SNARE complexes observed may be due to the interaction between SNARE-reconstituted vesicles of different size. To test this hypothesis, t-SNARE- and v-SNARE-reconstituted liposomes (proteoliposomes) of distinct diameters were used. Lipid vesicles of different sizes used in the study were isolated using published extrusion method.<sup>4,5</sup> The size of each vesicle population was further assessed using the AFM (Figure 1). AFM section analysis demonstrates the presence of small 40–50 nm diameter vesicles isolated using a 50 nm extruder filter (Figure 1A,B). Similarly, representative samples of large vesicles measuring 150–200 and 800–1000 nm were obtained using different size filters in the extruder. Such large vesicles are shown in the AFM micrograph (Figure 1C,D). Analysis of vesicle size using photon correlation spectroscopy further confirmed the uniformity in the size of vesicles within each vesicle population (data not shown).

The morphology and size of the SNARE complex formed by the interaction of t-SNARE- and v-SNARE-reconstituted vesicles of different diameter were examined using the AFM (Figure 2). In each case, the t-SNARE and v-SNARE proteins in opposing proteoliposomes interact and self-assemble in a circular pattern, forming pore-like structures. The interaction and arrangement of SNAREs in a characteristic ring pattern were observed for all populations of proteoliposomes examined (Figure 2A–D). However, the size of the SNARE complex was found to be dictated by the diameter of the proteoliposomes used (Figure 2). When small

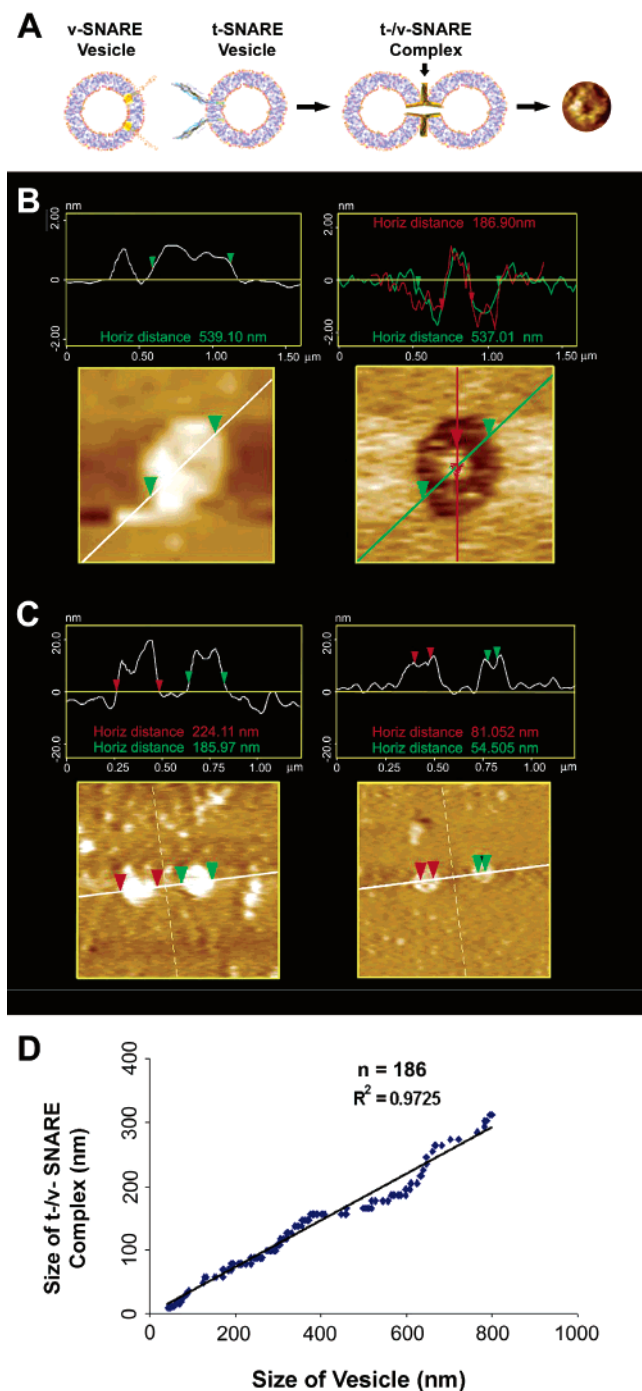


**Figure 1.** AFM micrograph of t-SNARE- and v-SNARE-reconstituted liposomes of different sizes. Note the ~40–50 nm vesicles (A, B), the ~150 nm (C), and the ~800 nm vesicle (D).



**Figure 2.** Representative AFM micrograph of t-/v-SNARE complexes formed when small (A, B) or large (C, D) t-SNARE- and v-SNARE-reconstituted vesicles interact with each other. Note the formation of different size SNARE complexes, which are arranged in a ring pattern. Bar = 20 nm. AFM section analysis (D) shows the size of a large SNARE complex.

(~50 nm) t-SNARE- and v-SNARE-reconstituted vesicles were allowed to interact, SNARE-ring complexes of ~20 nm diameter were generated (Figure 2A,B). With an increase in the diameter of proteoliposomes, larger t-/v-SNARE complexes were formed (Figure 2 C,D). A strong linear relationship between the size of the SNARE complex and vesicle diameter is demonstrated from these studies (Figure 3). The experimental data fit well with the



**Figure 3.** SNARE complex is directly proportional to vesicle diameter. Schematic diagram depicting the interaction of t-SNARE- and v-SNARE-reconstituted vesicles. At the extreme right, is a single t/v-SNARE complex imaged by AFM (A). AFM images of vesicles before and after their removal by the AFM cantilever tip, exposing the t/v-SNARE complex (B). Interacting t-SNARE and v-SNARE vesicles imaged by AFM at low (<200 pN) and high forces (300–500 pN). Note, at low imaging forces, only the vesicle profile is imaged (left C). However, at higher forces, the soft vesicle is flattened, allowing the SNARE complex to be imaged (right C). Plot of vesicle diameter versus size of the SNARE complex. Note the high correlation coefficient ( $R^2 = 0.9725$ ) between vesicle diameter and the size of the SNARE complex (D).

high correlation coefficient,  $R^2 = 0.9725$ , between vesicle diameter and SNARE complex size (Figure 3).

Unlike calculated values, which fundamentally assume vesicles to be nondeformable and hard spheres, our experimental data suggest that these artificial lipid vesicles, similar to secretory vesicles, are soft. Hence, once v-SNARE and t-SNAREs from opposing vesicles meet, the initial SNARE complex formed pulls the opposing bilayers closer to each other. As a consequence, vesicles become flattened, which then leads to an increase in contact area between the opposing vesicles. The result is a further increase in t/v-SNARE contacts, allowing the formation of larger SNARE-ring complexes. In the case of hard vesicles, flattening would be unlikely and, therefore, result in forming smaller SNARE-ring complexes. On the other hand, deformation of soft vesicles leads to an increase in contact area between the opposing bilayers and a resultant increase in Gibbs free energy. In an elastic membrane, the surface free energy is given by the equation:  $(1/2)k_a(\Delta A)^2/A_0$ , where  $k_a$  is the bending modulus,  $\Delta A$ , the increase in surface area, and  $A_0$ , the initial unstressed area.<sup>6</sup> Therefore, an increase in surface area results in an increase in the Gibbs free energy, and the spontaneous fusion between opposing bilayers becomes less probable.<sup>6–8</sup> Hence, large vesicles are less fusogenic than smaller vesicles. This would explain why neurons being fast secretory cells possess small 40–50 nm diameter vesicles for rapid and efficient fusion and release of neurotransmitters at the nerve terminal,<sup>9,10</sup> compared to a slow secretory cell, such as the exocrine pancreas, with larger (200–1200 nm diameter) secretory vesicles for the slow and prolonged release of digestive enzymes.<sup>11,12</sup>

In summary, we have demonstrated that the size of a self-assembled supramolecular SNARE protein complex can be controlled. This has implications in the regulated fusion of artificial lipid membranes, which may find use in the controlled delivery of lipid-encapsulated drugs and the transport of molecules.

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**Supporting Information Available:** Experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Supporting Information

**Preparation of t-SNARE and v-SNARE-associated liposomes of different sizes** - All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). A 10-mM lipid stock solution was prepared by mixing lipid solution in chloroform- DOPC (1,2-dioleoyl phosphatidylcholine): DOPS (1,2-dioleoyl phosphatidylserine) in 70:30 mol/mol ratios in glass test tubes. The lipid mixture was dried under gentle stream of nitrogen and resuspended in decane. The lipids were then suspended in buffer containing 10 mM Hepes-NaOH [pH =7.5] and 140 mM NaCl by vortexing for 5 min. at room temperature. Vesicles were prepared following sonication for 2 min, and extrusion using an extruder with membranes of different pore size. Vesicles of uniform size were prepared by a published extrusion method (1), using several passes of the liposomal solution through polycarbonate membranes of different pore size, in a LiposoFast extruder (Avestin, Ottawa). Vesicle size was determined using photon correlation spectroscopy and AFM. Proteoliposomes were prepared by gently mixing either the v-SNARE (VAMP2-His<sub>6</sub>, 153 µg/ml) or the t-SNAREs (syntaxin1A /SNAP25, 196 µg/ml) with liposomes (2,3), followed by three freeze-thaw cycles to enhance protein reconstitution at the vesicles membrane.

**Imaging vesicles and corresponding SNARE-complexes** - To prepare SNARE-complexes on mica for AFM studies, freshly cleaved mica disks were placed in a fluid chamber. Two hundred microliters of imaging buffer solution containing 140 mM NaCl, 10 mM HEPES, and 1 mM CaCl<sub>2</sub>, was placed at the center of the cleaved mica disk. Prior to deposition on mica, t-SNARE and v-SNARE reconstituted vesicles of approximately same size were gently mixed for 10 min. at room temperature. Both vesicle size and SNARE complexes were measured. SNARE complexes were also measured following depletion of lipids by solubilization using Triton X-100 (0.1% w/v). Ten microliters of the formed SNARE complex were added to a drop of buffer solution. The mixture was then allowed to incubate for 60 min at room temperature, prior to washing (x10), and imaging using the AFM.

The SNARE complexes shown in Fig. 2 were obtained both with and without lipid extraction. Fig. 2A,B are after lipid extraction, whereas Fig. 2C and D are SNARE complexes on lipid membrane. Once the SNARE ring complex is formed, there appears to be little difference in the structure following lipid extraction. Vesicle size and that of the corresponding SNARE complex was measured by the following method: (a). At low AFM imaging force, the vesicle profile is measured, and as the force is increased, the soft vesicle is flattened, and the underlying SNARE complex is revealed. (b). During repeated imaging of a fixed area, the attached vesicles are dislodged by the AFM cantilever tip, exposing the SNARE complex. Different size vesicles isolated using the extruder, provides only the upper limit of vesicle diameter. Thus the membrane having a pore size of 400 nm, used in the extruder, will result in a whole population of different size vesicles, up to a maximum of 400 nm, although the 300-400 nm size vesicles will be in the majority. However, to prevent vesicles beyond 2 µm in diameter (such very large vesicles tend to be too soft and flat), and to obtain vesicles of all different sizes, the extruder was employed.

**Atomic force microscopy** - AFM was performed on reconstituted lipid vesicles and SNARE complexes placed on the mica surface. Nanoscope IIIa AFM from Digital Instruments (Santa Barbara, CA) was used for these studies. Images were obtained both in the “contact” and “tapping” mode in fluid. However, all images presented in this manuscript were obtained in the “tapping” mode in fluid, using silicon nitride tips with a spring constant of 0.06 N/m, and an imaging force of <200 pN. To obtain high resolution images, imaging forces of up to 300-500 pN

were also used. Images were obtained at line frequencies of 1.98 Hz, with 512 lines per image and constant image gains. Topographical dimensions of SNARE complexes and lipid vesicles were analyzed using the software nanoscope IIIa4.43r8 supplied by Digital Instruments.

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