Complexin-1 Enhances the On-Rate of Vesicle Docking via Simultaneous SNARE and Membrane Interactions

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Abstract: In synaptic terminals, complexin is thought to have inhibitory and activating roles for spontaneous “mini” release and evoked synchronized neurotransmitter release, respectively. We used single vesicle-vesicle microscopy imaging to study the effect of complexin-1 on the on-rate of docking between vesicles that mimic synaptic vesicles and the plasma membrane. We found that complexin-1 enhances the on-rate of docking of synaptic vesicle mimics containing full-length synaptobrevin-2 and full-length synaptotagmin-1 to plasma membrane-mimicking vesicles containing full-length syntaxin-1A and SNAP-25A. This effect requires the C-terminal domain of complexin-1, which binds to the membrane, the presence of PS in the membrane, and the core region of complexin-1, which binds to the SNARE complex.

Ca2+-triggered, synchronized fusion of synaptic vesicles to the presynaptic plasma membrane underlies interneuronal communication. Proteins including neuronal soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), the Ca2+ sensor synaptotagmin-1, SM proteins, and complexin are critical for this process. Complexin is a small soluble protein (134 residues) that is mainly found in the presynaptic terminal. It binds to the SNARE complex and has both activating and inhibiting functions for fast synchronous release and spontaneous “mini” release. Moreover, overexpression of complexin in PC12 cells and chromaffin cells or expression as a fusion protein to synaptobrevin substantially diminished neurotransmitter release, suggesting an inhibitory role of complexin. In contrast, knockout of complexin isoforms from hippocampal neurons selectively impaired the synchronous component of exocytosis indicating a stimulatory role of complexins in late fusion steps.

The N-terminus (residues 1–27) of complexin-1 is critical for fast synchronized release, whereas the accessory α-helix (residues 27–48) plays a role in suppressing spontaneous release (Figure 1A). A central region of complexin (residues 48–70) binds to the groove between the synaptobrevin and syntaxin α-helices in the core part of the neuronal SNARE complex, which itself is a tight bundle of four α-helices; this central region appears to be critical for all functions of complexin. The C-terminal region (residues 70–134) has a role in synaptic vesicle priming, but the underlying molecular mechanism is unclear.

Figure 1. Single vesicle-vesicle docking assay: (A) Primary sequence domain diagrams of complexin-1 (Cpx), Cpx4M, and Cpx1-86 with functional annotations. (B) Schematic of our single vesicle-vesicle assay for measuring the docking probability between v- and t-vesicles. A saturated layer of DiD labeled t-vesicles (reconstituted with syntaxin-1A and SNAP-25A) was created by immobilization on the imaging surface through biotin-neutravidin tethers. The saturation and homogeneity of the layer of immobilized t-SNARE vesicles was assessed by red laser illumination at 633 nm. Free DiI labeled v-vesicles (reconstituted with full-length synaptobrevin-2 and synaptotagmin-1) were injected into the system in the presence or absence of complexin-1 for a defined incubation time period (25 s unless noted otherwise). Green laser illumination at 532 nm imaged the v-vesicles that were docked to immobilized t-vesicles. The mean diameter of the vesicles is 45 nm as determined by inspection of cryo-EM images of the vesicles (Figure S1). This setup is similar to that of ref 17.

In vitro biophysical studies revealed stimulatory effects of the central region and C-terminal regions of complexin in proteoliposome lipid-mixing experiments and inhibitory

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effects of full-length complexin in cell-based fusion, proteoliposome lipid-mixing assays, and synaptotagmin-binding competition experiments. However, the cell-based fusion assays examined relatively slow fusion events (minute time scale), and the lipid mixing experiments examined lipid mixing, rather than content mixing, the latter correlating with neurotransmitter release. Remarkably, we found that complexin dramatically increases the number of fast (msec) Ca2+ triggered fusion events between synaptic vesicle and plasma membrane mimics using a single-vesicle content mixing assay. At the lowest Ca2+ concentration that we tested (250 μM), the fusion probability increased from background levels to a substantial burst, in agreement with in vivo studies of synchronous release in neurons.

A “clamping” model of complexin has been proposed, in which complexin stabilizes the t-SNARE complex in an inhibitory conformation that blocks full complex formation with synaptobrevin, until a Ca2+ signal arrives, although the molecular mechanism of the release of the block remains unclear.

Synaptotagmin-1, a synaptic vesicle membrane-anchored Ca2+ sensor, plays an essential role for fast synchronous neurotransmitter release. The absence of full-length synaptotagmin-1 in many previous in vitro studies (or, instead, the use of the soluble C2AB domain of synaptotagmin-1) may thus explain the differences between physiological observations and these particular experiments. Here we studied the effect of complexin-1 on the on-rate of docking between vesicles that mimic synaptic vesicles, containing both full-length synaptotagmin-1 and synaptobrevin-2, and vesicles that mimic the plasma membrane, containing both syntaxin-1A and SNAP-25A. We found that complexin-1 enhanced the on-rate of docking. However, this enhancement was critically dependent on the presence of the membrane-binding C-terminal domain of complexin, in agreement with recent in vivo data. In the absence of the C-terminal binding domain, the on-rate of docking was reduced in the presence of complexin. We note that both properties of complexin also depend on the interaction between the central region of complexin and the SNARE complex and on the presence of PS in the membrane.

We immobilized DiD labeled "t-vesicles" (proteoliposomes with reconstituted full-length syntaxin-1A and SNAP-25A) on a polyethylene glycol (PEG) coated imaging surface (Figure 1B). We subsequently injected a solution of DiI labeled "v-vesicles" (proteoliposomes with reconstituted full-length synaptobrevin-2 and synaptotagmin-1) in the presence or absence of 10 μM complexin-1 for a defined incubation time period (see online Supporting Information, SI, for details). The v-vesicles mimic synaptic vesicles, while the t-vesicles mimic the plasma membrane. At the end of the incubation period (25 s, unless mentioned otherwise), unbound v-vesicles and complexin-1 molecules were removed by buffer exchange (SI and Figure S2). Using green laser illumination, we then counted the average number of DiI-labeled v-vesicles per imaging area (50 × 100 μm2) that remained bound to t-vesicles. Since our protocol produces a homogeneous and saturated surface-layer of immobilized t-vesicles, the number of fluorescent spots arising from DiI labeled v-vesicles is proportional to the probability that a v-vesicle docks to a surface-tethered t-vesicle during the incubation period, and it is proportional to the duration of the incubation period since the off-rate is rather slow. By design, this experiment measures a non-equilibrium property that depends on the kinetics of the interaction between v- and t-vesicles. Below saturating conditions, the docking probability within the incubation time period is approximately related to the on-rate of docking between free v-vesicles and immobilized t-vesicles. The number of vesicles that dock to a saturated surface within a defined time period has been measured in previous single-particle experiments. Figure 3 illustrates the kinetic character of our measurement by using two different incubation time periods (see further discussion below).

As previously noted, we included full-length synaptotagmin-1, in contrast to previous liposome-based studies that examined the effect of complexin in the presence of SNAREs only. Remarkably, in our experiments complexin-1 significantly increased the docking probability by ~60% rather than reducing it (Figure 2A). We next tested if the enhancement in docking probability by complexin-1 depends on the interaction with the SNARE complex. We employed the "4M" mutant (R48A, R59A, K69A, and Y70A) of complexin-1, Cpx4M, that significantly weakens the interaction with the SNARE complex. The v-vesicle docking on-rate in the presence of the Cpx4M mutant was statistically identical to the case without complexin-1 (Figure 2A). Thus, the enhancement of docking by complexin-1 in the presence of both SNAREs and synaptotagmin-1 depends on this particular interaction with the neuronal SNARE complex.

As a further control, the SNARE dependence of the effects for wild-type complexin and its mutants was tested by preincubating the immobilized v-vesicles with a large excess (20 μM) of the soluble synaptobrevin-2 fragment (residues 1–96) (Figure 2B). In all cases, the pre-incubation of t-vesicles with the synaptobrevin fragment diminished docking of v-vesicles in the absence or presence of complexin as well as its mutants. This result can be explained by the sequestration of all
t-SNARE complexes by the soluble synaptobrevin fragment, preventing trans-SNARE complex formation and, hence, vesicle docking.

Since docked v-vesicles remain associated with the immobilized t-vesicles during the short imaging period, the observed docking probability in our experiments should be proportional to the on-rate of the association reaction. Since this probability would be independent of the duration of the incubation period, one would expect that the docking probability ratios between different conditions to be independent of the incubation period as well. Indeed, for a shorter incubation period, the ratio between two particular conditions is close to that of the longer period (Figure S3). Moreover, as shown in Figure S4A, the relative docking probability pattern was also independent of the v-vesicle concentration. As an additional control, the docking probability ratios are approximately independent of the v-vesicle concentration (Figure S4A). Moreover, the majority of docked v-vesicles are involved in single v-/t-vesicle pairs as assessed by fluorescence intensity profiles (Figure S4B).

We next tested if the enhancement of the docking probability by complexin-1 depends on its C-terminus. Surprisingly, the C-terminally truncated construct of complexin-1, Cpx1−86, reduced the docking on-rate (Figure 2A). Similarly, in the absence of full-length synaptotagmin-1, Cpx1−86 also reduced the docking probability (Figure 3). Moreover, using a liposome flotation assay, we found that the C-terminus is critical for binding of complexin-1 to synthetic membranes with a lipid composition similar to that of synaptic vesicles (Figure 4). Finally, the presence of phosphatidylserine (PS) in the v-vesicle membrane is essential for the docking enhancement by complexin (Figure 5). This result is consistent with a previous finding that PS is a binding partner to complexin.9,21

Previous studies based on an ensemble in vitro lipid mixing assay proposed a potential fusion promoting role of the C-terminus of complexin-1,9,10 which contrasts a recent in vivo study suggesting that C-terminus is important for vesicle docking, but not fusion.7 Here, we report a critical role of the complexin C-terminus for enhancing the on-rate of docking between vesicles that mimic synaptic vesicle and the plasma membrane, consistent with the in vivo results. How can one resolve this apparent contradiction between previous in vitro bulk lipid-mixing experiments and our single-vesicle results? Fluorescence correlation spectroscopy (FCS) lipid-mixing experiments revealed that the rate-limiting step of an ensemble in vitro lipid mixing assay is the docking step itself rather than the fusion reaction.22 Thus, a factor that promotes vesicle docking would also enhance subsequent lipid mixing and fusion events, rather than affecting the fusion kinetics itself.17,22 Single-vesicle assays are capable of discriminating between effects related to docking, hemifusion, and complete fusion, so they are not subject to the limitations of certain bulk lipid-mixing experiments.

Our single-vesicle results suggest that the C-terminus of complexin plays a key role in enhancing the docking on-rate of synaptic vesicles. Most recently, a study from Rizo’s group showed that the C-terminus of complexin is important for resisting synaptotagmin replacement.23 A large body of work has focused on the SNARE-interacting part of complexin-1, the accessory helix, and the N-terminal region for roles in synchronizing fast release and suppressing...
spontaneous release. Together with previous studies, an important functional role of C-terminal membrane-binding region of complexin has been uncovered and warrants further study to decipher the underlying molecular mechanism.

At variance with many previous in vitro studies, we included both full-length neuronal SNAREs and synaptotagmin-1 in order to provide better mimics of both synaptic vesicles and the plasma membrane. Compared to the soluble C2AB fragment of synaptotagmin-1, membrane-anchoring of full-length synaptotagmin-1 works in a different manner for efficient Ca\textsuperscript{2+} triggering. We thus recommend that future studies of Ca\textsuperscript{2+} triggered fusion should always, at minimum, include both full-length neuronal SNAREs and synaptotagmin-1 in addition to neuronal SNAREs, as was already done in recent studies.

## ASSOCIATED CONTENT

Supporting Information
Detailed experimental methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Complexin-1 enhances the on-rate of vesicle docking via simultaneous SNARE and membrane interactions

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Experimental Methods:

Protein expression and purification. Full-length rat syntaxin-1A, full-length SNAP-25A, full-length synaptobrevin-2, full-length synaptotagmin-1 were expressed, purified as described previously \(^1\) with modifications \(^2\). Briefly, his-tagged syntaxin-1A and synaptobrevin-2 were expressed in C43 (DE3) cells \(^3\) and SNAP-25A was expressed in BL21 (DE3) cells (Novagen). The proteins were purified by Ni\(^{2+}\)-nitrilotriacetic acid (NTA) sepharose (Qiagen) affinity chromatography and further purified by size exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare) in buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM TCEP and 110 mM octyl-\(\beta\)-D-glucoside (OG, ANATRACE). No OG was used for SNAP-25. Synaptotagmin-1 was expressed in Sf9 insect cells (Invitrogen) and purified by Ni\(^{2+}\)-NTA
affinity chromatography (Qiagen), followed by size exclusion chromatography on a Superdex 200 16/600 column (GE Healthcare) and further purified by anion-exchange chromatography on a Mono-S 5/50 column (GE Healthcare). His-tags were removed from syntaxin-1A, synaptobrevin-2, and SNAP-25A with TEV protease, or from synaptotagmin-1 with PreScission protease (GE Healthcare, Uppsala, Sweden), and proteins further purified as previously described.

Soluble rat synaptobrevin-2 (residues 1-96) was expressed in BL21 (DE3) with an N-terminal TEV cleavable hexa-His tag. The protein was purified by Ni-NTA affinity chromatography using standard procedures and buffers (Qiagen), digested overnight with TEV protease, and further purified by size exclusion chromatography using a HiLoad Superdex 200 16/600 column that was pre-equilibrated with 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA and 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

Full-length rat complexin-1 (referred to as Cpx), and the “4M” mutant (R48A, R59A, K69A, Y70A) of complexin-1 (referred to as Cpx4M) were purified as previously described. The C-terminally truncated mutant of complexin-1 (residues 1-86, referred to as CPX1-86) was expressed as a glutathione S-transferase (GST) fusion protein from pGEX-KT (GE Healthcare) in BL21 (DE3) and purified on a glutathione-agarose column (GE Healthcare). The protein was eluted from a glutathione column by digestion with Thrombin (HTI), concentrated, and further purified by size exclusion chromatography on a HiLoad Superdex 200 16/600 column that was pre-equilibrated with 20 mM Hepes-KOH pH 7.5, 100 mM NaCl, and 0.5 mM TCEP.
Vesicle reconstitution of syntaxin-1A, synaptobrevin-2, and synaptotagmin-1. Syntaxin-1A, synaptobrevin-2, and synaptotagmin-1 were reconstituted into vesicles as previously described \(^2\) except that distinct lipid dyes were added to v- and t-vesicles: 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate, DiIC18(5) (DiD)/ 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate, DiIC18(5) (DiI) labels were added during the reconstitution of v- and t- SNARE vesicles, respectively \(^4\). The particular composition followed our previous work \(^1\): t-vesicles consisted of Brain Total Lipid Extract, supplemented with 20 mol% cholesterol, 3.5 mol% phosphatidyinositol-4,5-bisphosphate (PIP2), 0.2 mol% biotinylated phosphatidylethanolamine (PE), and 2 mol% DiD (all lipids from Avanti polar lipids) and v-vesicles consisted of phosphocholine (PC):PE: phenylserine (PS):Cholesterol:DiI in a ratio of 46:20:12:20:2.

As previously described \(^4\), lipid films were dissolved in 110 mM OG buffer and tag free proteins (synaptobrevin-2/synaptotagmin-1 and syntaxin-1A for v- and t-vesicles, respectively) were added at a protein to lipid ratio of 1:200. For t-vesicles, a large excess of soluble SNAP-25A (five times the concentration of syntaxin) was added to the protein-lipid mixture in order to reduce the possibility of formation of dead-end 2:1 syntaxin/SNAP-25 complexes. Detergent free buffer (20 mM HEPES, pH 7.4, 90 mM NaCl, 1 % 2-mercaptoethanol) was added to the protein-lipid mixture until the detergent concentration was at its critical micelle concentration. The vesicles were purified with a CL4B desalting column and dialyzed overnight with Bio-beads SM2 (Bio-rad) in detergent-free Vesicle Buffer (20 mM HEPES, pH 7.4, 90 mM NaCl, 20 µM
EGTA, 1% 2-mercaptoethanol). For the reconstitution of v-vesicles with both full-length synaptobrevin-2 and synaptotagmin-1, a 4.6:1 protein ratio was used in order to mimic the observed protein concentration in synaptic vesicles.

**PEG surface preparation.** Details of the preparation for PEGylation of surfaces are described in reference 4. Briefly, cleaned or new quartz slides and glass coverslips were incubated with 150 ml acetone (Fisher) solution containing 5 ml Tris(hydroxymethyl)aminomethane (Fisher) for 30 min. After washing with acetone, air-dried quartz slides and glass coverslips were assembled into a sandwich structure with PEG solution (10 mg Biotin-PEG-SVA and 90 mg mPEG-SVA dissolved in 0.1 M sodium bicarbonate, pH 8.5) in between; note that we used mPEG-SVA instead of mPEG-SCM at variance with the protocol published in Ref. 4 since mPEG-SVA produces a better surface coverage. After incubation in the dark for 2-10 hours, the quartz slides and glass coverslips were disassembled and washed with deionized water, air dried, and stored at -20 °C. Quality controls included checking the homogeneity of each surface preparation (Figure S6) and tests for non-specific binding (Figure 2B).

**Single vesicle-vesicle docking experiments.** A saturated layer of DiD-labeled t-vesicles was immobilized on an imaging surface via biotin/neutravidin interactions. Specifically, the DiD-labeled t-vesicle solution (described above) was diluted 10 × with Vesicle Buffer. 100 µl of the diluted t-vesicle solution was injected into the sample chamber and incubated for 30 min, followed by buffer exchange (1 × 200 µl vesicle buffer) for 6 sec. Next, the DiI-labeled v-vesicle solution (described above) was diluted 50-100 ×. 100 µl of diluted free-floating DiD-labeled v-
vesicle solution was injected into the sample chamber in the presence or absence of wildtype or mutant complexin-1 (10 µM). After an incubation period, unbound v-vesicles were removed by buffer exchange (2 × 200 µl vesicle buffer for ~20 sec) (Figure S1). 20 µM EGTA was included in all solutions for elimination of free Ca^{2+} ions.

We determined the optimum incubation period (25 sec) by trial and error, i.e., providing a sufficiently large number of docked vesicles below the density limit (~1000 in a 50 x 50 µm^2 field of view) in order to optically resolve individual vesicles. In addition, we performed intensity distribution analyses (Figure S4 and Figure S5) that allowed us to conclude that mostly single v-vesicles are docked to the surface (as opposed to multiple v-vesicles docked to one surface-immobilized t-vesicle).

Sample slides with 5 channels were monitored in a wide-field TIR fluorescence microscope (Nikon) using an electron multiplying charge-coupled device (CCD) camera (iXon+ DV 897E, Andor Technology). A program (smCamera) written in C++ was used for data acquisition and analysis (available from Taekjip Ha, University of Illinois). 10 images were taken at random locations within each channel on the quartz slide. Details regarding software, slide assembly, and imaging protocols are described in reference 4.

We confirmed that the t-vesicle-covered surfaces were saturated and produced a homogeneous distribution for each surface preparation with red laser excitation (633 nm) of the DiD-labeled
immobilized vesicles, as observed in a separate DiD channel on particular slide (Figure S5). As previously reported, more than 1000 vesicles could be immobilized with this method. Our preparation of a reproducible, homogeneous, and saturated surface of immobilized t-vesicles ensures that the number of docked DiI labeled v-vesicles is directly related to the docking probability.

For each set of comparisons between different conditions and/or mutants (Figures 2B, 2C, and 3) the same protein preparations and surface preparations (quartz slide with immobilized vesicles), and incubation times were used, and the conditions were run in separate channels on the same slide. The relative differences and ratios were statistically similar for different protein preparations.

**Membrane binding experiment.** Protein-free vesicles were made using the method described above, containing PC, PE, PS lipids and cholesterol at a molar ratio of 48:20:12:20 (the same ratio as used to reconstitute the v-vesicles, except that the 2% ratio corresponding to the lipid-dyes was added to the PC ratio). The vesicles encapsulated 0.1 M sucrose in order to increase the density of the vesicles. 1 µM Cpx or Cpx1-86 were incubated along with these vesicles (0.8 mM total lipid) at 4 °C for 2 hours. The vesicles were separated from unbound complexin by centrifugation at 230,000 G for 45 min. Membrane pellets were suspended in 2/5 the volume of 1X Laemmli sample buffer containing DTT, and supernatant fractions were diluted with 1/2 volume of 3x sample buffer. 5 µl aliquots were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on an AnyKD gel (Biorad) and were blotted to a nitrocellulose membrane which was then probed with a polyclonal antibody raised against
human complexin-1 (Abcam, product ab102761). The blot was developed using standard procedures with a horseradish peroxidase coupled anti-Rabbit IgG (Peirce, product #31460).
Figure S1. Vesicle diameter distribution. A and B: Cryo-EM images that were used for determining the vesicle diameter distribution. V- and t-vesicles were mixed for 30 minutes before flash freezing in liquid nitrogen using similar procedures as described in ref. 2. Vesicles were picked by inspection of the cryo-EM images (indicated by a faint green dot in the center of a particular vesicle), and the diameter measured as an average over multiple center sections across the vesicle. Scale bars are 100 nm. C: A histogram showing the distribution of vesicle diameters of both t- and v-vesicles combined. The black line is a Gaussian fit to the observed diameter distribution; the mean diameter is 45 nm.
**Figure S2.** Experimental flow. Immobilization of DiD labeled t-vesicles, buffer exchange, incubation with Dil-labeled v-vesicles in presence of absence of complexin-1, and buffer exchange. The incubation period with Dil-labeled v-vesicles that we used in our experiments was in the range 7~25 sec. Significantly longer incubation periods resulted in too many docked vesicles that prevented optical separation of single vesicles (the theoretical optical separation limit for our setup is 237.5 nm, using the Airy disk approximation with a numerical aperture of 1.2 and a wavelength of 570 nm).
Figure S3. Dependence of the number of docked v-vesicles on the incubation time. A 100× dilution of DiI v-SNARE vesicles was used in this experiment. Because the injection method is a manual procedure, about 2~3 sec pass when switching to different buffers. Thus, there is some uncertainty in estimating the incubation time, affecting the total counts for the experiments. However, the ratio of the average counts between the two experimental conditions (SNAREs, synaptotagmin-1 & Cpx vs. SNAREs, synaptotagmin-1, & Cpx) should not be affected by this uncertainty. Indeed, the ratio of the two conditions (SNAREs, synaptotagmin-1 & Cpx vs. SNAREs, synaptotagmin-1 & Cpx) is 2.42 and 2.35 for incubation times of 25 and 7 sec, respectively. Error bars are SEM from 10 random imaging locations in the same sample channel.
Figure S4. Docking experiments at lower v-vesicle concentration. (A) Number of docked v-vesicles for wildtype and mutants of complexin-1. The protocol described in Methods and Figure 1 was used. The v-vesicle concentration was half of that of the experiments shown in Figure 2. (A) The pattern of the docked vesicle counts is similar to that shown in Figure 2, i.e., the relative docking count is roughly independent of the v-vesicle concentration. (B) Distribution of the fluorescence intensity of all observed fluorescent spots for each of the four conditions, corresponding to the experiments shown in panel (A). The intensity distributions have maxima in the range of 0.4-1.2 (a.u.), suggesting that primarily single v-/t-vesicle pairs occur, i.e., there is a very low probability that two or more v-vesicles are docking to one t-vesicle.
Figure S5. Fluorescence intensity distribution of fluorescent spots for the experiments shown in Figure 5. The intensity distributions have maxima in the range of 0.4-1.2 (a.u.), suggesting mostly single v-/t-vesicle pairs are observed.
Figure S6. Fluorescent images of surface-immobilized DiD-labeled t-vesicles using red laser excitation. The images indicate that our Method generates saturated layers with more than 1000 immobilized t-vesicles, minimizing the possibility of non-specific surface interactions. Indeed, Figure 2B shows that there is only a low probability of non-specific binding.
References


