Biophysical Journal, Volume 98

Supporting Material

Direct visualization of large and protein-free hemifusion diaphragms

Jörg Nikolaus, Martin Stöckl, Dieter Langosch, Rudolf Volkmer, and Andreas Herrmann

Direct visualization of large and protein-free hemifusion diaphragms

Jörg Nikolaus, Martin Stöckl, Dieter Langosch, Rudolf Volkmer and Andreas Herrmann

SUPPORTING MATERIAL

Fig. S1

Further examples for sequences of fusion between GUVs made of DOPC:DOPE:DOPS (3:1:1, mol/mol/mol), containing either 1 mol% Rh-LLV16-Rh (indicated by an *arrow*) or 1 mol% N-NBD-PE. Pairs of GUVs were imaged by fluorescence microscopy (excitation filter 470-490 nm, emission filter >510 nm (LP)) at 25°C. Upon addition of 6 mM Ca^{2+} fusion was monitored. The first image corresponding to t = 0 refers to the last snapshot before alterations of the adhesion region between two GUVs were observed. Magnifications of selected images are shown. Arrows indicate the dimension of the developing HD. In the last images the HD ruptures very likely at junction site of the three bilayers at the HD periphery and retracts to the other side (indicated by an *arrow*). Bar 5 µm.

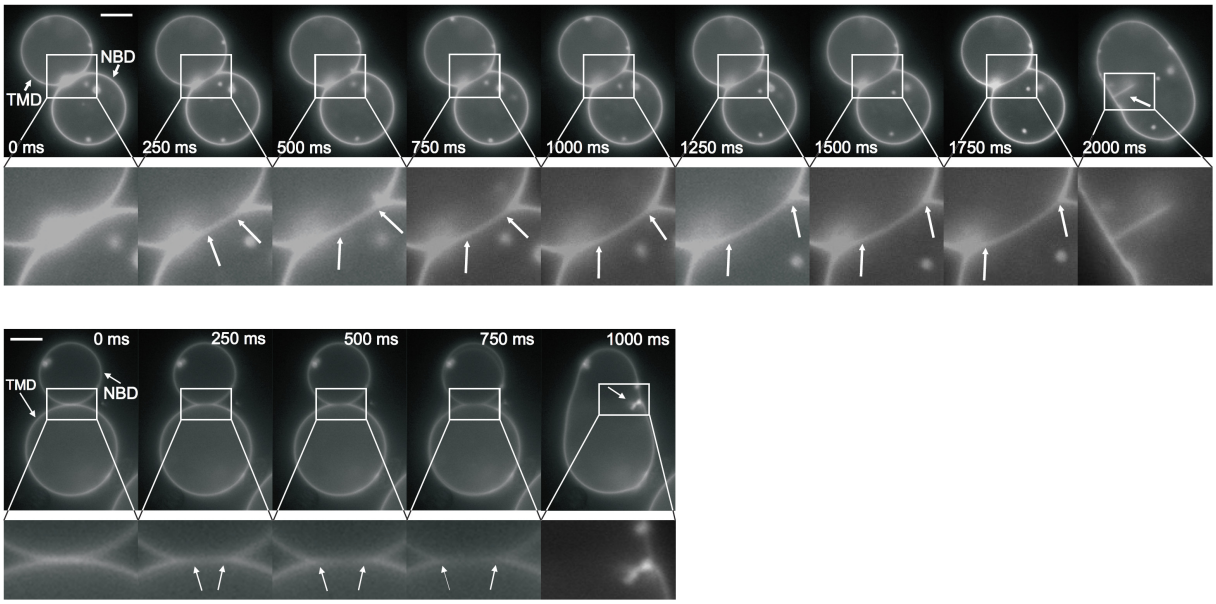

Fig. S1

Fig. S2

Adhesion regions without sequestered peptides. Examples of GUV pairs for which peptides (Rh-HA) are not sequestered from the adhesion region. (*A*) Pair of N-NBD-PE labeled GUV and of peptide (1 mol%) containing GUV. Lateral distribution of Rh-labeled peptide, N-NBD-PE and overlay are shown. Note N-NBD-PE is not redistributed to the peptide-containing GUV (cf. Fig 2 A(II)). (*B* to *D*) Both GUV contain 1 mol % (*B* and *C*) or 5 mol% peptide (*D*). Bar 5 µm.

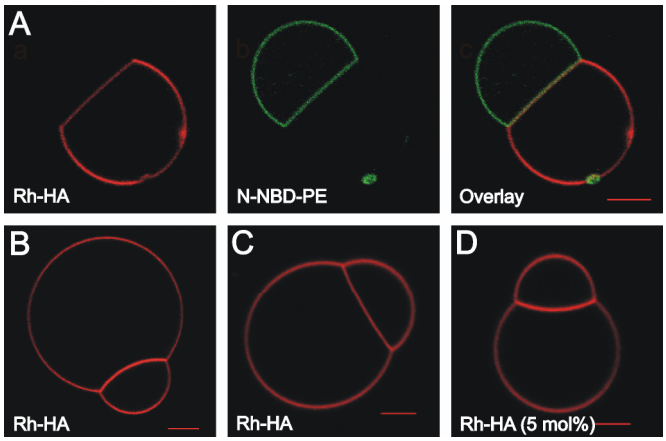

Fig. S2

Fig. S3

Sequestering of C6-NBD-PC upon HD formation. Outer membrane leaflets of GUVs were labeled by addition of C6-NBD-PC prior to addition of Mg^{2+}. (*A*): Sketch of C6-NBD-PC localization. (I) If an HD is formed C6-NBD-PC is sequestered from the forming HD. (II) C6-NBD-PC remains in the adhesion region when it is formed by two separated bilayers.

(*B*): Addition of 2 mM Mg^{2+} led to the adhesion of the GUVs with sequestered Rh-HA peptides. Images of rhodamine and NBD fluorescence of two attached GUVs both containing the Rh-HA peptide. Peptides as well as the C6-NBD-PC are sequestered from the HD. Bar 5 µm.

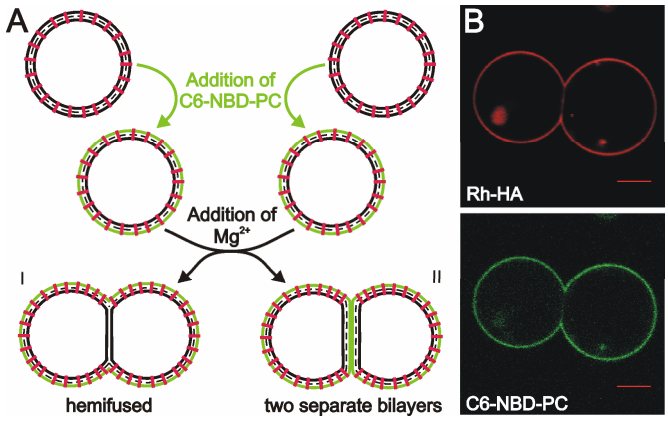

Fig. S3

Fig. S4

Fluorescence recovery after photobleaching (FRAP)

FRAP measurements were performed with the same confocal setup as described above. Laser intensity was established at 488 nm to photobleach about 80% of the fluorescence of the NBD lipid analogue. FRAP was performed in the equatorial plane of a GUV bleaching an upright membrane area of about 7 µm × 1 µm. Subsequently, scanning of the bleached area was continued with low laser intensity for about 90 seconds. Recovery kinetics was fitted to $F(t) = F_0\tau + F_\infty t / (t + \tau)$ which is an approximation of the theoretical recovery curve, where t is the time after bleaching, F(t) is the fluorescence as a function of time, F_0 is the fluorescence immediately after bleaching, F_∞ is the amount of fluorescence recovery, and τ is the time of half-maximal recovery (Axelrod et al., 1976, Kwon et al., 1994). Fitting procedures yield accurately fitted experimental curves. Calculation of the diffusion coefficient (D) was done using the equation: $D = (\gamma/4)\omega/\tau$ where γ is related to the bleaching depth and is approximately 1.45 under the experimental conditions (Yguerabide et al., 1982), ω is the bleached area, and τ is the calculated time for half-maximal recovery. We are aware that performing FRAP in an upright membrane area may not necessarily be comparable with standard FRAP experiments on horizontal membranes, but appropriate for comparison of the two situations, diffusion within the HD and outside of this region, respectively. The mobile fraction (M_f) was calculated using: $M_f = (F_\infty - F_0)/(1 - F_0)$.

FRAP shows similar diffusion properties of fluorescent lipid analogue in the GUV membrane outside the HD (*blue*, n=22) and in the HD of two hemifused GUVs (*red*, n=14), respectively. (*A*)**:** Diffusion coefficient D (µm^2/s) and (*B*)**:** Mobile fraction. Bars represent the mean ± SD of at least 14 measurements. (*C*)**:** Two selected fluorescence recovery graphs for FRAP measurements in GUV membrane outside the HD (*blue*) and within HD (*red*), respectively.

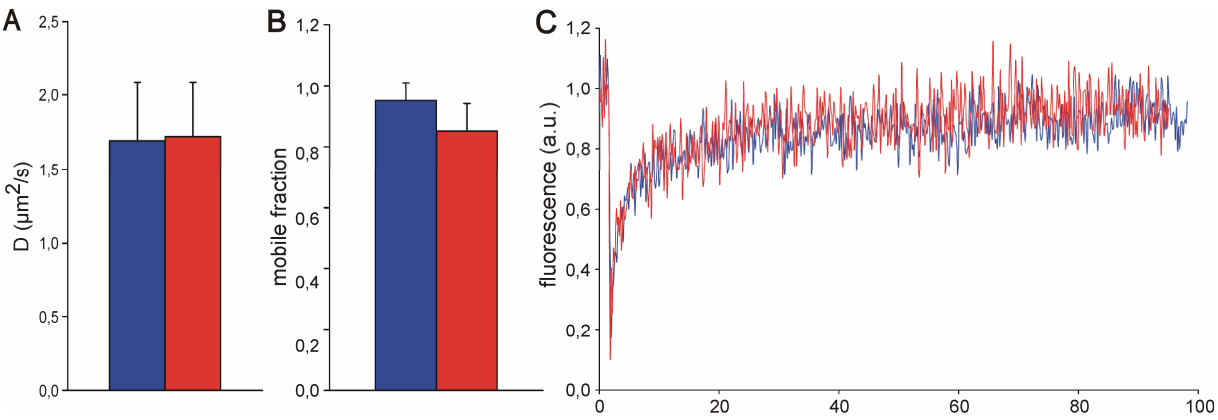

Fig S4

Calculation of the relative surface area of HDs

Fig. S5

Estimation of the relative surface area of HD. Due to a decrease of membrane tension upon hemifusion of GUVs they flatten upon settling onto the coverslip. Based on the resulting geometry the relative surface area of HDs of the total surface of 50 attached GUV pairs with sequestered peptides (see Table 1) was estimated.

(*A*): Sketch of two hemifused GUVs settled onto a coverslip. The top view shows the diameters of both GUVs (d_1, d_2) and of the HD (d_{HD}). GUVs were approximated by half spheres.

(*B* and *C*): Confocal image (*B*) and Z-Stack (*C*) of a GUV pair containing the peptide Rh-HA and N-NBD-PE after addition of 2 mM Ca^{2+}. Note, that the upper part of the large GUV was not imaged and therefore omitted (*C*). Bar 5 µm.

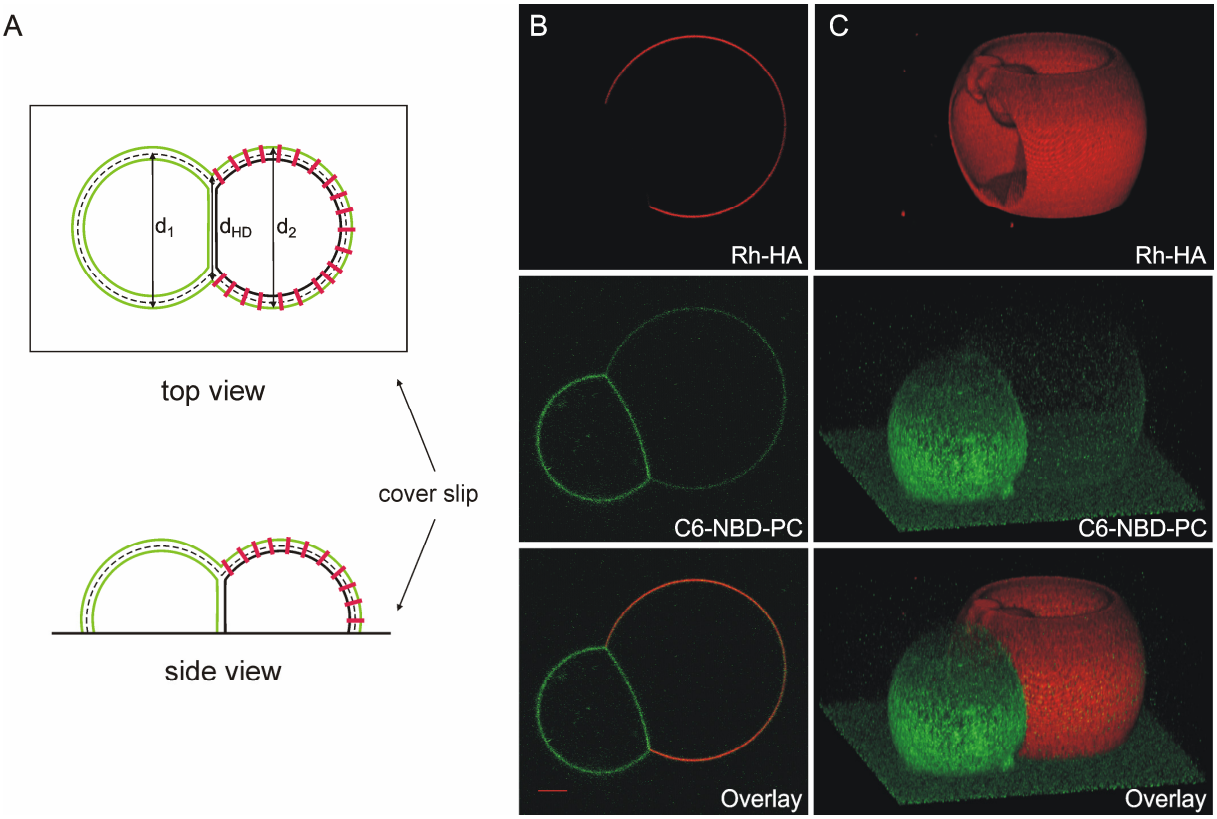

Fig. S5

Fig. S5

Calculation

Equations for calculation are given below. Data are given in the table. We estimated a relative surface area of about 9%. This provides an upper estimate, since GUVs were approximated by half spheres. However, as evident from images (Fig. S5 B) the diameter of the flat area could be smaller in comparison to the GUV diameters d_1 and d_2, respectively.

	mean ± SEM
$A_{1,S} = \frac{1}{2}\pi d_1^2$	389 ± 39 µm^2
$A_{1,P} = \frac{\pi}{4}d^2$	194 ± 20 µm^2
$A_{HD} = \frac{1}{2}\frac{\pi}{4}d^2$	93 ± 13 µm^2
$A_{2,S} = \frac{1}{2}\pi d_2^2$	1127 ± 140 µm^2
$A_{2,P} = \frac{\pi}{4}d^2$	563 ± 70 µm^2
$\%\ HD = \frac{2A_{HD}}{A_{1,S}+A_{2,S}+A_{1,P}+A_{2,P}-2A_{HD}} \times 100$	**8.7 ± 0.4 %**

$A_{1,S}$ – Area of the half sphere of GUV 1
$A_{1,P}$ – Area of the plane in contact with the coverslip of GUV 1

A_{HD} – Area of the HD (estimated as a half circular area)

$A_{2,S}$ – Area of the half sphere of GUV 2
$A_{2,P}$ – Area of the plane in contact with the coverslip of GUV 2

% HD – fraction of the HD area in the total area of GUV 1 and GUV 2

Table 1 Calculation of HD size for DOPC:DOPE:DOPS (3:1:1)

	d_1	d_2	d_{HD}	$A_{1,S}$	$A_{1,P}$	A_{HD}	$A_{2,S}$	$A_{2,P}$	$\frac{2A_{HD} \quad x100}{A_{1,S}+A_{1,P}+A_{2,S}+A_{2,P}}$
	/ µm	/ µm	/ µm	/ µm²	/ µm²	/ µm²	/ µm²	/ µm²	%
	12	22	15	226	113	88	760	380	11.9
	32	55	40	1608	804	628	4752	2376	13.2
	14	38	16	308	154	101	2268	1134	5.2
	20	53	24	628	314	226	4412	2206	6.0
	12	35	16	226	113	101	1924	962	6.2
	26	28	21	1062	531	173	1232	616	10.1
	17	24	16	454	227	101	905	452	9.9
	14	22	12	308	154	57	760	380	7.1
	18	21	15	509	254	88	693	346	9.8
	14	23	15	308	154	88	831	415	10.3
	15	16	12	353	177	57	402	201	10.0
	14	42	16	308	154	101	2771	1385	4.4
	13	42	14	265	133	77	2771	1385	3.4
	16	18	10	402	201	39	509	254	5.7
	22	22	18	760	380	127	760	380	11.2
	22	26	19	760	380	142	1062	531	10.4
	13	17	12	265	133	57	454	227	10.5
	17	22	15	454	227	88	760	380	9.7
	12	34	14	226	113	77	1816	908	5.0
	11	32	14	190	95	77	1608	804	5.7
	16	30	18	402	201	127	1414	707	9.3
	12	22	11	226	113	48	760	380	6.4
	25	42	25	982	491	245	2771	1385	8.7
	15	25	15	353	177	88	982	491	8.8
	14	16	13	308	154	66	402	201	12.5
	11	20	12	190	95	57	628	314	9.2
	12	11	7	226	113	19	190	95	6.2
	11	24	12	190	95	57	905	452	6.9
	15	25	16	353	177	101	982	491	10.0
	16	25	14	402	201	77	982	491	7.4
	13	18	12	265	133	57	509	254	9.7
	16	22	17	402	201	113	760	380	13.0
	10	18	10	157	79	39	509	254	7.9
	16	24	16	402	201	101	905	452	10.3
	16	18	14	402	201	77	509	254	11.3
	12	14	11	226	113	48	308	154	11.9
	11	27	12	190	95	57	1145	573	5.6
	20	22	16	628	314	101	760	380	9.7
	19	24	17	567	284	113	905	452	10.3
	10	16	11	157	79	48	402	201	11.3
	10	28	11	157	79	48	1232	616	4.6
	9	18	11	127	64	48	509	254	10.0
	10	20	10	157	79	39	628	314	6.7
	17	18	14	454	227	77	509	254	10.7
	13	21	13	265	133	66	693	346	9.2
	7	8	6	77	38	14	101	50	10.6
	13	20	11	265	133	48	628	314	7.1
	6	17	7	57	28	19	454	227	5.0
	17	18	14	454	227	77	509	254	10.7
	22	43	24	760	380	226	2904	1452	8.2
Mean	**15.0**	**24.9**	**14.7**	**389**	**194**	**96**	**1127**	**563**	**8.7**
SEM	**0.7**	**1.4**	**0.8**	**39**	**20**	**13**	**140**	**70**	**0.4**

Fig. S6

Dependence of HD size on TMD concentration. For increasing Rh-HA peptide concentration in GUVs a decrease in the HD size was observed. Plotted values are (mean ± SEM) µm of HD size versus mol % of TMD peptide concentration reconstituted into GUVs. 0 mol% TMD value stands for hemifusion of two GUVs both labeled with N-NBD-PE and without TMD peptides. In the latter case formation of HD was detected by measuring and comparing the fluorescence intensity of N-NBD-PE in the different GUV regions (see Results).

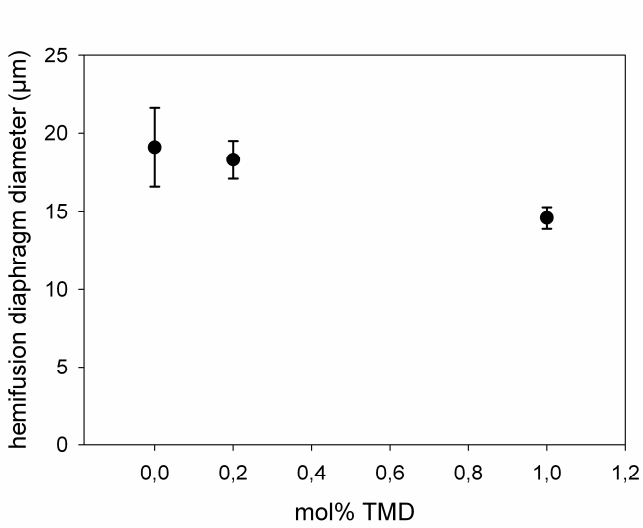

Fig. S6

Reconstitution and labeling of HA protein

To visualize reconstitution of HA protein, virus was preincubated for 2 h at RT in the dark with TMR, added form 5 mM Me_2SO stock to molar excess of 10 over HA. Uncoupled TMR was removed by washing labeled virus in phosphate buffered saline (PBS, pH 7.4) and harvested by centrifugation at 52 000 × g. SDS-PAGE analysis revealed that only influenza virus HA was labeled (data not shown). Virosomes were prepared according to Papadopulos et al., 2007. Triton X-100-solubilized influenza virus (strain X31) was mixed with a Triton X-100-solubilized lipid mixture of DOPC:DOPE:DOPS (3:1:1, mol/mol/mol) at lipid/protein ratio of 20 (w/w) and incubated for 1 h. To remove detergent and to generate virosomes 1 g of SM2 BioBeads (Bio-Rad) per 70 mg of Triton X-100 were added and rotated at 4°C. After 12 h the same amount of BioBeads was added for another 4 h. The turbid suspension was withdrawn carefully from the beads and washed two times with PBS and collected by centrifugation (55 000 × *g*, 1h, 4°C) to remove remaining detergent.

GUVs with reconstituted viral protein were generated according to Girard et al., 2004. Virosomes were diluted with distilled water to 0.4-0.8 mg/ml lipid, 0,02 g sucrose/g lipid was added to protect proteins during the following dehydration (Doeven et al., 2005) and finally 50 µl of this suspension was deposited onto each ITO slide or titanium plate. Dehydration was achieved by placing the slides in a sealed chamber containing a saturated NaCl solution overnight. From here, formation of GUVs follows the electroformation method described above.

References

Axelrod D, Koppel DE, Schlessinger J, Elson E, Webb WW (1976) Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys J* 16:1055-1069.

Kwon G, Axelrod D, Neubig RR (1994) Lateral mobility of tetramethylrhodamine (TMR) labelled G protein alpha and beta gamma subunits in NG 108-15 cells. *Cell Signal* 6:663-679.

Yguerabide J, Schmidt JA, & Yguerabide EE (1982) Lateral mobility in membranes as detected by fluorescence recovery after photobleaching. *Biophys J* 40: 69-75.

Papadopulos A, Vehring S, Lopez-Montero I, Kutschenko L, Stöckl M, Devaux PF, Kozlov M, Pomorski T, Herrmann A (2007) Flippase activity detected with unlabeled lipids by shape changes of giant unilamellar vesicles. *J Biol Chem* 282: 15559-15568.

Girard P, Pécréaux J, Lenoir G, Falson P, Rigaud JL, Bassereau P (2004) A new method for the reconstitution of membrane proteins into giant unilamellar vesicles. *Biophys J* 87**:** 419-429.

Doeven MK, Folgering JHA, Krasnikov V, Geertsma ER, Bogaart G, Poolman B (2005) Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles. *Biophys J* 88**:** 1134-1142.