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ARTICLE

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Short-range specific forces are able to induce hemifusion

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Abstract Working with pure lipidic systems (giant unilamellar vesicles, 10–150 μm in diameter) as models for biological membranes, we have considered possible structures of the contact area of two adherent membranes by investigating the diffusion of fluorescent lipid analogues from one vesicle to another. Two bilayers in close contact can almost be seen as a lamellar structure in equilibrium. This is the usual configuration of two adherent vesicles, in which the interbilayer distance is estimated to be 3 nm. We have increased the attraction between the membranes by either adding depletion forces or by using a trick, inspired from the interaction between nucleic bases in nucleosides (herein adenosine and thymidine). The nucleosides were attached to the polar head of amphiphilic molecules that behave like phospholipids and were incorporated in the model membrane. The extra attraction between two membranes, resulting from base pairing, strongly decreased the interbilayer distance down to about 1 nm. This change of the water content induced lipid rearrangements, which could also be viewed in terms of a phase transition at low water content. These rearrangements were not observed in the case of depletion forces. We conclude that the introduction of an additional attractive force in the system modifies the equilibrium state, leading to a drastic change in the membrane behavior, which will tentatively be related to hemifusion.

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Introduction

Interactions between biological membranes have been extensively studied for decades (Evans et al. 1991; Zimmerberg et al. 1993; Chernomordik et al. 1995). This problem is of major interest in biology as it is relevant to adhesion and fusion processes. Even though a large number of proteins involved in these processes have been identified, the mechanisms of fusion are far from being clearly understood. To figure out the role of proteins, model lipid systems are of common use (Novick and Hoekstra 1988; Weber et al. 1998). These models take advantage of the absence of undefined proteins or proteins which could interfere. They are based on the very similar physical properties of artificial lipid assemblies and biological membranes, as long as the physicochemical parameters are well chosen. Nevertheless, authors mainly focus on the proteins, and the possible role of lipids in these phenomena has been only scarcely considered (Devaux et al. 1993; Chernomordik et al. 1995; Mouritsen and Kinnunen 1996).

Amphiphile self-assemblies have been thoroughly investigated and liposomes widely used as model membranes (Lis et al. 1982; Marra and Israelachvili 1985; Larche et al. 1986; Porte et al. 1988). Their physical properties are strongly size dependent. Whereas small unilamellar vesicles (a few tens of nanometers in diameter) have very high curvature energy and tension, giant unilamellar vesicles [GUVs, up to few hundreds of micrometers in diameter (Angelova et al. 1992; Mathivet et al. 1996)] resemble biological membranes as they have cell size and similar elastic properties.

Many authors have opened the field of predicting the forces responsible for adhesion and quantifying them, using giant vesicles (Evans 1980; Helfrich and Servuss 1984; Servuss and Helfrich 1989), lamellar phases (Lis

et al. 1982) or supported bilayers (Marra and Israelachvili 1985; Wolfe et al. 1991).

Recently, membrane hemifusion between GUVs has been observed, using strong electrostatic attraction between oppositely charged vesicles (Pantazanos and MacDonald 1999). Here we present work in which hemifusion is related to dehydration of the polar head. The equilibrium state of two adherent vesicles can be varied by modulating the weak adhesion forces through local hydrogen bonds. The equilibrium distance between the vesicles is quantified and determined by the force balance. An increase in attraction leads to a decrease in this distance. This closer contact, even though not optically observable, must correspond to a lower amount of water in the contact zone. This water content has a lower limit at which a phase transition must occur (Seddon 1990).

The aim of our study is to probe this local phase transition, investigate the structure of the final phase with regard to hemifusion, and finally compare our model to the hemifusion intermediate step proposed in biological systems. For that purpose, we used electroformed GUVs and investigated attraction forces ranging from electrostatic screening to strong hydrogen bonding, including depletion forces. Hydrogen bonding was achieved through recognition between two complementary nucleosides.

Intervesicle forces

The events we monitored are due to a balance of intervesicles forces. They mainly consist of (1) attractive forces, i.e. van der Waals and, if relevant, other extra attractive forces (see below) and (2) repulsive forces, i.e. double layer electrostatic repulsion, Helfrich (thermal undulation) (Helfrich and Servuss 1984; Servuss and Helfrich 1989) and short-range repulsion forces (hydration, protrusion, steric hindrance).

In a glucose solution, electrostatic repulsion is predominant, since, even with "neutral" lipids, such as DOPC, membranes have been shown to bear a small charge (Pincet et al. 1999). As this repulsion may prevent adhesion, it has to be screened by salt addition.

The extra attractive forces consist of either depletion forces (Evans and Needham 1988) or molecular recognition (H-bonds). Depletion is obtained by adding high molecular weight dextran to the system. Hydrogen bonds are obtained by inserting lipid derivatives of nucleosides, interacting through the previously described and characterized molecular attraction between adenosine and thymidine (Pincet et al. 1994).

Materials and methods

Materials

The functionalized lipids with thymidine (DOT) or adenosine (DOA) as a headgroup (Fig. 1) were obtained by coupling the unprotected nucleosides to 2-(1,3-dioleyloxy)propylhemisuccinic acid using a modified DCC/DMAP method (Lebeau et al. 1992). The fluorescent 1-acyl-2-[12-(NBD-amino)stearoyl]-*sn*-glycerol-3-phosphocholine (NBD-PC) was synthesized according to Colleau et al. (1991). Dioleoyl-*sn*-phosphatidylcholine (DOPC) and dextran were purchased from Sigma.

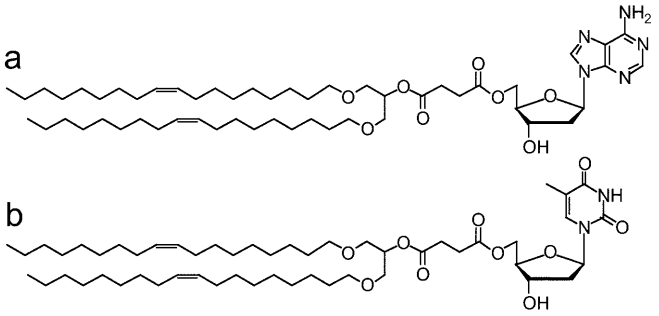

Fig. 1 **a** DOA and **b** DOT molecules

Preparation of GUVs

GUVs were produced by an a.c. electric-field-controlled swelling of a lipid film, essentially as described previously (Mathivet et al. 1996), an improvement of the method originally introduced by Angelova et al. (1992) [for a detailed list of references see also Bucher et al. (1998)]. A particular advantage of the electroformation method is that it produces unilamellar vesicles in a very high yield (Mathivet et al. 1996). Four different GUV compositions were used, as listed in Table 1. Populations 2 and 4 are fluorescently labeled. We checked that the vesicles containing DOA or DOT were still unilamellar (EM experiments, data not shown). The incorporation of the functionalized lipids is symmetrical, their chains are exactly the same as the ones for DOPC and their compression isotherms are almost identical (Lis et al. 1982; Pincet et al. 1994); therefore the spontaneous curvature is not a relevant parameter in our experiments. GUVs were formed in a swelling solution containing 346 mM glucose/3 mM sodium azide/15 mg mL^{-1} dextran (MW 150,000). GUVs were withdrawn from the formation chamber with a microsyringe and kept at 4 °C under argon in a 360 mM glucose/3 mM sodium azide solution, hereinafter called the sedimentation solution; as the latter is less dense than the swelling solution, the GUVs settle down in the preserving tube.

Iso-osmotic solution

Since GUVs are very sensitive to osmotic pressure, we checked very carefully the osmolarity of each solution. The swelling solution osmolarity was 365 ± 3 mosm and the sedimentation solution osmolarity was always fixed slightly higher, 380 ± 3 mosm, to prevent excessive swelling which could induce tension in the membrane.

Observation of liposomes

Samples were observed by either epifluorescence or phase contrast optical microscopy, using an inverted microscope (Zeiss IM35).

Table 1 Composition of the four populations of GUVs that were used (% w/w)

	DOPC	DOA	DOT	NBD-DOPC
1. DOT	95	–	5	–
2. DOA*	90	5	–	5
3. DOPC	100	–	–	–
4. DOPC*	95	–	–	5

The fluorescence was excited by an argon laser tuned at 488 nm (blue). The laser beam was used at low intensity to avoid thermal damage and to minimize photobleaching; the maximum wattage was 50 μW over an area of 400 μm^2. The objective lens was ×100 (Zeiss Phaco 3). The observation chambers were petri dishes, the bottoms of which had been partly replaced by a glass coverslip stuck with silicone. After sample deposition, another coverslip was placed on top to prevent water evaporation and to create a flat interface for direct illumination, which is a minimum requirement for phase contrast observation. The above-described chamber had a thickness of 0.8 mm due to the petri dish wall thickness. The maximum volume was about 250 μL. When the samples had to be kept over hours, water was added to the petri dish (not in contact with the sample), and the cover was sealed with parafilm.

Results

In the experiments, we mix two GUV populations, one of them being fluorescently labeled. Four sets of experiments were conducted, as indicated in Table 2: control experiments, depletion experiments, A-T experiments and A-T depletion experiments. Typically, 20 μL of each GUV suspension were deposited in a 20 μL drop of sedimentation solution.

After sedimentation, a layer of GUVs covers the bottom of the chamber but does not adhere because of the electrostatic repulsion. Depending on the sample, this layer can be more or less dense. However, a denser region is always found around the center of the sample. The fluorescent and non-fluorescent GUVs are randomly distributed.

Addition of salt (NaCl approximately 200 mM, final concentration 30 mM) creates a gentle flow, which allows the GUVs to moderately diffuse on the bottom of the chamber, increasing the collision probability. As expected, after this addition, adhesion occurs in the four sets of experiments. The aggregation of the vesicles is very fast: an equilibrium is reached within a minute. A typical adhesion event consists in the aggregation of two GUVs. As the adherent vesicles are randomly distributed, aggregates can be composed of either two vesicles of the same type or one vesicle of each type, as observed by fluorescence. Fluorescence microscopy observation focused on aggregates composed of two different types of vesicle, in order to follow the fluorescence distribution. In the case of the A-T experiments and A-T depletion experiments, and only in these cases, adhesion is always followed by fluorescence redistribution over the surface of the whole edifice within 30 min (Figs. 2 and 3).

Table 2 Description of each kind of experiment: types of GUVs that were mixed and composition of the solution in which the experiment was carried out. Experiments were repeated at least 30 times for each case

Solution composition (380 mosm)	Type of GUV	
	DOPC/DOPC*	DOT/DOA*
30 mM NaCl	Control experiment	A-T experiment
30 mM NaCl + 15% w/w dextran (MW 298,000)	Depletion experiment	A-T depletion experiment

This phenomenon is never observed in control or depletion experiments. The time elapsed between adhesion and the onset of fluorescence diffusion is variable (about 10 min), but the fluorescence becomes homogeneous on the second vesicle within a few minutes. The final fluorescence intensity is always lower on the initially non-fluorescent liposome compared to that of the initially fluorescent one.

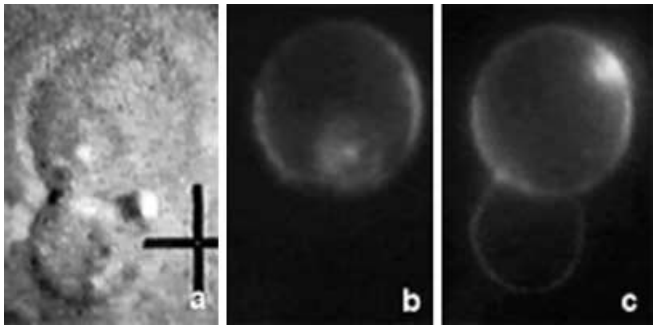

Fig. 2a–c Two different populations of GUVs, DOT and DOA*, are brought into contact in the presence of 25 mM NaCl. Three pictures of the same vesicles are presented (**a** and **b** are taken at the same time; **c** is taken 30 min later). Adhesion is first observed (**a** phase contrast microscopy, **b** fluorescence microscopy) and is followed by the redistribution of the fluorescence over the surface of the whole edifice (**c**). The *cross* represents 10 μm

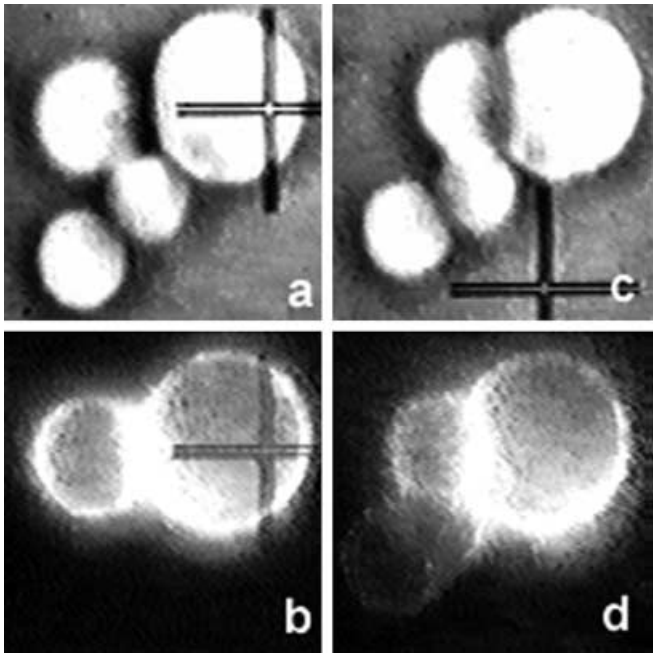

Fig. 3a–d Two different populations of GUVs, DOT and DOA* are brought into contact in the presence of dextran (MW 298,000) + 25 mM NaCl. Adhesion is first observed (**a** phase contrast microscopy, **b** fluorescence microscopy) and is followed by the redistribution of the fluorescence over the surface of the whole edifice, along with an increase in the contact areas (**c** phase contrast microscopy, **d** fluorescence microscopy). Note that when dextran is added, the optical contrast is inverted (**a** and **c**). The *cross* represents 10 μm

In the case of the control and depletion experiments, fluorescent and non-fluorescent GUVs can still be observed in the chamber, even after 20 h when the sample is kept in a moist argon atmosphere. Under the same conditions, all the vesicles are totally fluorescent after 2 h in the A-T experiments and A-T depletion experiments.

Discussion

The adhesion step is observed under the same conditions in all the experiments. As already described, it is mainly due to van der Waals forces when salt is added to the glucose solution (Evans and Metcalfe 1984). These attractive forces are predominant at long distance (a few tens of nanometres) and H-bonds (between DOA and DOT) cannot form at such a long distance. Thus, in our control experiments the vesicles reach an equilibrium state when the distance corresponds to that where the van der Waals attraction and, if relevant, depletion forces are balanced by short-range repulsion. That state is a real equilibrium in our control and depletion experiments. In contrast, in the A-T recognition experiments the latter situation corresponds to a transient state as the membranes are close enough (~3 nm) to allow the formation of H-bonds between the A and T moieties (Pincet et al. 1997). The nucleoside derivatives are only present at 5% in the membrane but can freely diffuse laterally, i.e. toward the contact region. Thus, more and more A-T bonds can form, decreasing the mean intervesicle distance. At that stage, the water content becomes too small to fully hydrate the polar headgroups, bilayers become unstable, and the local stress in the outer layers is relaxed by transition to another structure.

More quantitatively, following Evans (1991) numerical procedure, we can estimate the equilibrium distance between pure DOPC membranes to be 3 nm (Fig. 4) in the control experiment and 2.9 nm in the depletion experiment (numerical parameters are given in the figure captions). This is equivalent to a dozen water layers, and corresponds in the DOPC/water phase diagram to a water content of about 35% (w/w). When A-T interactions have to be taken into account, the extra attractive force can be calculated from other experimental data (Pincet et al. 1994; Boland and Ratner 1995) (see Appendix). The calculated equilibrium distance then decreases down to 1 nm (Fig. 4). The water content has to be divided by 3, which corresponds to about 10 water molecules per lipid in the interaction area. It is known that below that hydration number, the structure of the lipid assemblies has to be modified (McIntosh and Magid 1993). At room temperature, DOPC adopts another bilayer structure with a reduced molecular area. In our configuration, since the molecular area has to remain constant, this is no longer conceivable, and the modification can only consist of a transition to a nonlamellar structure. The exact scheme of this phase is difficult to guess; however, the outer layers of the vesicles that are now in too close a contact must have merged. In this model, the inner layers are not under the same stress, and may not be concerned by the rearrangement of the outer layers.

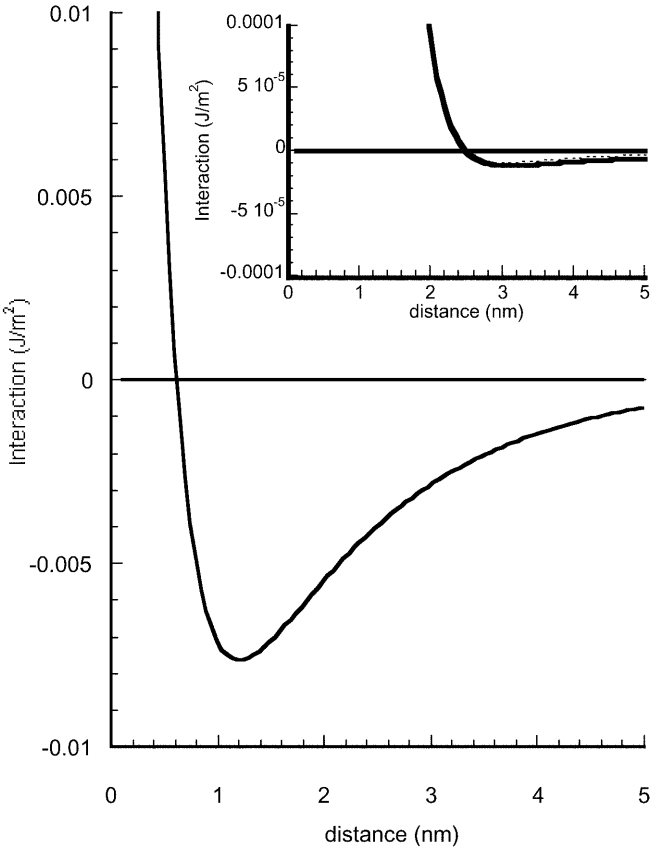

Fig. 4 Energy of interaction between two vesicles, numerically calculated as described by Evans (1991), without nucleosides (*insert*) and in the presence of nucleosides. The short-range repulsion [0.16 exp(–*d*/0.29)] J m^{-2} [where *d* (in nm) stands for the intermolecular distance between the two membranes] and the Hamaker constant (6×10^{-21} J) were taken from Lis et al. (1982)

This is in good agreement with our experimental observations, in which the coalescence of the outer layers is evidenced by diffusion of the fluorescence throughout the whole edifice while keeping a membrane between the two compartments. More precisely, the inhomogeneity of the fluorescence supports this hypothesis. Even though there are some methodological difficulties for a quantitative analysis of the fluorescence (the vesicles are not always in the same plane, perpendicular to the observation direction), our results are compatible with the expected ratio between the less fluorescent part and the other (Fig. 5). For vesicles of the same size, this ratio is 1/3. Taking into account the difference in size between the interacting vesicles, the exact expected value remains within the experimental error. The time scale of this fluorescence redistribution also fits the expected time for lipid diffusion in DOPC lipid bilayers (1 μm^2 s^{-1}) (Cribier et al. 1990).

This phenomenon, in which two membranes become connected, without any exchange between their inner medium, has also been described in a biological system

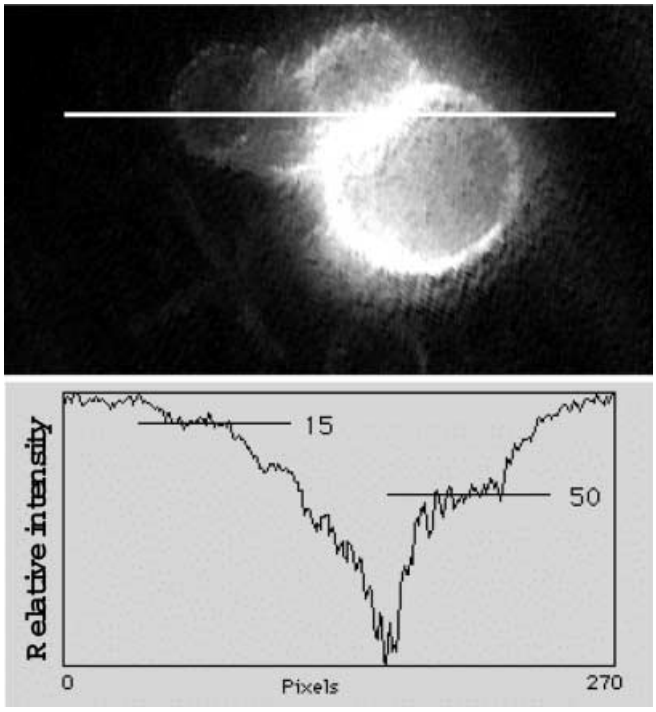

Fig. 5 Tentative quantification of fluorescence repartition between both vesicles. Owing to the halo, only the more extreme vesicles are relevant. The measured ratio is $15/50 \approx 0.3$, as expected for hemifusion (see text). The contrast has been enhanced in the picture, leading to saturation in some areas while the profile was directly obtained from the row signal. The profile analysis has been made using "NIH image" software. The intensity profile (*graph*) is drawn following the *white line* on the picture

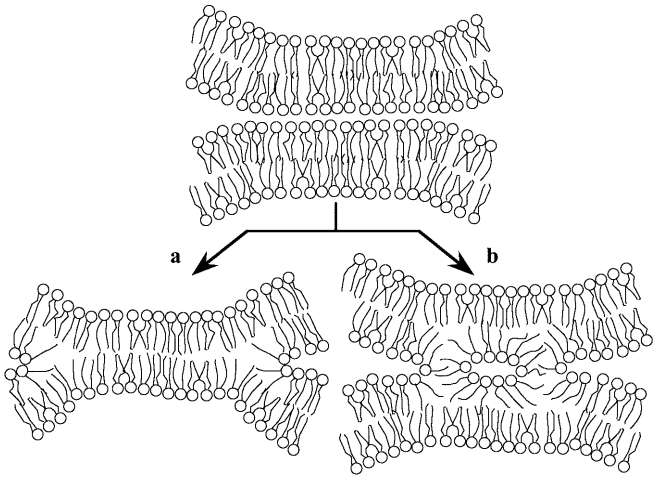

Fig. 6 Two representations of hemifusion: *a* the usual scheme where two layers fuse; *b* the inverted micelles intermediate (IMI) in our system; IMI or any similar topological structure (see text) is more likely to be the correct scheme because of volume/area constraints

(Melikyan et al. 1995) and is named hemifusion. A usual scheme for hemifusion is shown in Fig. 6 and can possibly occur when one of the vesicles is much smaller than the other one. In our experiments, this scheme is not realistic because of the area constraint: the total membrane area must remain the same throughout the process. Thus the exact structure of the membrane at the molecular level must be more complicated. Theoretical studies on the lamellar-inverted hexagonal phase transition, prompted Siegel (1986a, 1986b) to propose a role for the H_{II} phase in membrane fusion. In this model, inverted micelle intermediates (IMIs) are drawn as an intermediate state in the fusion process (Fig. 6). When many IMIs are present on the same hemifusion diaphragm, the putative structure resembles the stalk hypothesis (Chernomordik and Zimmerberg 1995). Other models have been proposed. The flip-flop described by Marchi-Artzner et al. (1996), consisting of a transverse diffusion where the hydrophobic tails have to cross the water gap, is much faster than the usual flip-flop (within a single bilayer and with a characteristic time of minutes instead of hours), and these authors attribute the accelerated lipid transverse redistribution to an unusually close proximity of the two vesicles. We do not want to question any of these different points of view as we think they all lead to the same result. All these descriptions correspond to a static view: both flip-flop or inverted micelles are likely to be part of the dynamic process. The clue of these models is the existence of tight contacts between the two membranes, which allow lipids to diffuse from one outer layer to the other.

Even though complete fusion through a non-expanding pore (Chanturiya et al. 1997) cannot be completely ruled out, the observation of Pantazanos and MacDonald (1999) indicates that when fusion is obtained between two GUVs, it is immediately followed by disruption of the whole structure. We have also observed such disruptions.

As intermembrane distances and intermembrane forces are strongly correlated, the hemifusion can also be investigated in term of forces. The force involved is typically a few tens of piconewtons; acting upon the area of one molecule (i.e. 0.65 nm^2), this will lead to an extremely high pressure indeed: a few hundred atmospheres. One can imagine that by pushing two vesicles on each other at such a high pressure, hemifusion could occur. Depletion forces will never be able to generate such pressure: a high polymer concentration such as the one we used brought the vesicles only 0.1 nm closer. Moreover, neither vesicles nor common cells would stand up to such a high pressure. Therefore the force has to be understood in terms of a local force creating a local stress, high enough to locally destabilize the outer layers. This could explain why, in biological systems, fusion is never globally induced on a cell, but locally induced at the molecular level by proteins, these latter playing the same role as the H-bonds in our system. For instance, the SNAREs (Weber et al. 1998) and the HA fusion peptide (influenza virus) (Chernomordik et al. 1995; Weissenhorn et al. 1997), described in recent models, are peptides which are anchored to the membranes and their partial structural changes bring the membranes into tight contact. A major difference with our system is that the hydrophobic parts of the peptides are always

embedded through the bilayer; therefore both leaflets are involved and fusion can be achieved.

Conclusion

We interpret the diffusion of the fluorescence from the initially fluorescent GUV to the former non-fluorescent GUV in the following way: (1) vesicles adhere because of van der Waals and depletion forces; (2) A-T H-bonds form, leading to a closer contact; (3) the decrease in the thickness of the intermembrane water layer destabilizes the bilayer structures, leading to hemifusion.

The role of the H-bonds in our system can be compared to that of fusion proteins (SNARE, HA fusion peptide). We have shown that hemifusion can be achieved in pure lipid systems, as long as an additional attractive force is locally applied. One can imagine that complete fusion could be achieved in a pure lipid system by mechanically coupling both leaflets or endogenous electroporation as suggested by Rosenheck (1998).

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Appendix: quantification of the A-T attraction

The interaction between DOA and DOT has been previously quantified in bilayer experiments (Pincet et al. 1994; Boland and Ratner 1995). From these data the interaction energy can be approximated as $2.1 \times 10^{-2} \exp(-d/1.5)$ J m^{-2}, where d (in nm) stands for the intermolecular distance between the two complementary nucleosides. In our system, DOA and DOT represent only 5% of the total lipids. One may think that this expression has to be strongly reduced in our case. In fact, base stacking has been observed in monolayers of either DOT or DOA, leading to the formation of very large domains when compared to the decay lengths of the involved interactions (Perez et al. 1998). Therefore one might consider that the above expression gives a good estimation of the local attraction in the neighborhood of the functionalized lipid domains.

Once again, following Evans (1991) numerical procedure and adding this extra term, the global interaction can be computed, leading to a different equilibrium contact distance: 1.2 nm (Fig. 4). Note that this distance is an average distance between the functionalized domains; when DOA and DOT are bound, they are indeed in molecular contact.

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