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Influenza hemagglutinin mediated fusion of membranes containing poly(ethylene-glycol) grafted lipids: new insights into the fusion mechanism

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Abstract The influence of a hydrophilic layer covering the membrane on influenza hemagglutinin (HA) mediated fusion was investigated using membranes containing poly(ethylene-glycol) grafted phosphatidylethanolamine (PEG-2000-PE). Steric inhibition of HA-membrane interactions by these lipids affected virus fusion (half-maximal inhibition at 0.8 mol% for lipids with 114 ethylene glycol residues, or at 3.2 mol% for 45 residues (PEG-2000-PE), concentrations at which the PEG moieties adopt a random coil structure). Reconstituted viral membranes containing 3 mol% PEG-2000-PE retained 40% of their fusion activity. Therefore, efficient fusion is possible with membranes completely covered by a hydrophilic layer of several nanometers, and fusogenic virosomes containing PEG-PE are feasible.

Key words: Influenza virus; Membrane fusion; Poly(ethylene-glycol); Hemagglutinin

1. Introduction

Influenza virus enters its host cell by endocytosis, followed by fusion between the viral and the endosomal membrane (for reviews see [1,2]). The initial binding to sialic acid residues on the plasma membrane and fusion are mediated by the viral integral membrane protein hemagglutinin (HA). Induction of a conformational change in HA by the low endosomal pH moves the hydrophobic N-terminus of the HA2 subunit, the 'fusion peptide', from the interior of the stem of the HA trimer towards the outside [3,4]. Experiments using liposomes as target membranes for viral fusion have yielded many insights into the fusion mechanism, since quantitative and kinetic fusion assays can be used and the composition of the membrane can be varied at will [5]. For example, it has been shown that the fusion peptide is inserted in the hydrophobic interior of the liposomal membrane just before fusion [6,7].

Influenza's biological target membranes, cellular plasma membranes, are surrounded by a hydrophilic glycocalyx formed by the oligosaccharide chains of proteins, lipids, and proteoglycans, presenting a potential barrier for the fusion peptide. In order to determine how the presence of such a layer might affect binding and fusion of the virus, we have produced liposomes containing poly(ethylene-glycol) grafted lipids (PEG-PE). These grafts form a non-adsorptive, hydrophilic layer, the thickness of which can be varied at will by the choice of the polymer size and the PEG-PE concentration.

PEG-PE lipids were developed to render liposomes 'invisible' to the cells of the reticuloendothelial system (RES) (reviewed in [8]), by preventing adsorption of proteins and adhesion to cells, as well as by preventing aggregation of liposomes and membrane fusion, as was shown for fusion between various artificial lipid membranes, in the absence of fusion proteins, in vitro [9–11]. Reconstituted influenza virus membranes might serve as vehicles for gene therapy [12], but they are potentially easily cleared in vivo by cells of the RES. Therefore, we have also tested if fusion induced by these preparations would still be possible if the reconstituted membranes contained PEG-PE.

Incorporation of PEG-PE into a membrane allows the precise calculation of the thickness of the hydrophilic layer covering a membrane. At low densities, the lipid grafted polymers probably adopt a random coil structure. In this 'mushroom' concentration range, the adjacent polymers do not overlap, but at higher densities (the 'brush' regime), the membranes are completely covered with the polymers, and lateral overlap between neighboring chains causes these to stretch out [13]. Thus, using the scaling theory of de Gennes the maximal thickness of the hydrophilic layer surrounding the membrane at a given concentration can be calculated. These predictions were corroborated by experimentation [14], and physical differences between the regimes confirmed experimentally in some cases [15–17].

We show here that, although PEG-PE lipids inhibit fusion mediated by HA whether they are present in the target membrane or in the reconstituted viral membrane, efficient fusion with membranes covered by a considerable thickness (several nanometers) of hydrophilic material occurs.

2. Materials and methods

2.1. Liposome preparation

Multilamellar vesicles without PEG-PE were produced by resuspension of dry lipid films of egg phosphatidylcholine, egg phosphatidylethanolamine (both from Avanti Polar Lipids, Birmingham, AL, USA), gangliosides (Sigma, St. Louis, MO, USA, type III from bovine brain, estimated molecular weight 1500 g/mol) at a molar ratio of 6:3:1 in buffer containing 145 mM NaCl, 2.5 mM HEPES, and 1 mM EDTA, pH 7.4. Liposomes with PEG-PE contained the above lipids in the same proportions, but additionally various amounts of 1,2-distearoyl-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000] (PEG-2000-PE, contains 45 ethylene glycol repeats) or PEG-5000-PE (114 repeats), both from Avanti, were present in the dry lipid film. These relatively long acyl chains were chosen because they are more effective anchors for PEG conjugates than shorter chains [9,18]. The suspension was frozen and thawed five times and large unilamellar vesicles were made from the multilamellar vesicles by extrusion through 0.1 μm defined-pore polycarbonate filters (Nucleopore, Pleasanton, CA, USA) [19]. After extrusion, remaining multilamellar liposomes were removed by centrifugation. Phospholipid phosphate was determined according to Böttcher et al. [20]. Incorporation of the PEG-PE lipids in the liposomes was checked by thin layer chromatography.

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2.2. Virus

The X-31 recombinant strain of influenza A virus (from plaque C-22 [21]) was grown for us by the Schweizerisches Serum- und Impfinstitut (Bern, Switzerland) in the allantoic cavity of embryonated eggs, and purified, handled and stored essentially as described before [22]. Viral phospholipid was extracted according to Folch et al. [23] and phospholipid phosphate was determined according to Böttcher et al. [20].

2.3. Fusion and binding measurements

Fusion between virus and labeled liposomes was measured with a resonance energy transfer assay [24]. Labeled liposomes contained 0.6 mol% each of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE). *N*-NBD fluorescence was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, using a 515 nm long-pass filter between cuvette and emission monochromator [25] and with continuous stirring in a thermostatted cuvette holder of an SLM 8000 D or a Jasco spectrofluorimeter. All measurements were carried out in buffer containing 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES, 1 mM EDTA at pH 5.1 or 7.4. For calibration of the fluorescence scale, the initial residual fluorescence intensity was considered to be zero and the intensity at infinite probe solution 100%. The latter value was obtained by lysis of the liposomes with 0.5% (v/v) Triton X-100 and corrected for the quenching of NBD by Triton as described [24].

To measure the binding of virus to liposomes, virus was incubated with fluorescently labeled liposomes at pH 5.1, 0°C, or pH 7.4, 0°C for 15 min, after which the mixture was neutralized and spun for 30 min at $16\,000\times g$. Pellet and supernatant were separated, and after addition of Triton X-100, 0.5% (v/v) to the samples, the *N*-NBD fluorescence was measured as described for fusion above. The amount of liposomes coprecipitated with the virus was calculated and corrected for the amount of liposomes pelleted in the absence of virus [26,27].

2.4. Reconstitution of viral membranes

Reconstitution of influenza virus was carried out according to [28]. Briefly, a concentrated pellet of influenza virus (1 μmol of viral phospholipid) was solubilized in 0.7 ml of 75 mM of the detergent octaethyleneglycol mono dodecyl ether ($C_{12}E_8$) (Fluka, Buchs, Switzerland) in buffer (145 mM NaCl, 2.5 mM HEPES, pH 7.4) for 30 min, 4°C. After removal of the ribonucleoprotein complex by centrifugation at $160\,000\times g$ for 30 min, the supernatant was added to a dry lipid film composed of a quantity of *N*-NBD-PE and *N*-Rh-PE corresponding to 0.6 mol% each of total phospholipid in the reconstituted membrane and sometimes also of PEG-2000-PE or PEG-5000-PE, and vortexed vigorously. The mixture was added to pre-washed BioBeads SM-2 (20 mg dry beads/70 μl) and shaken at 1400 rpm in an Eppendorf shaker for 1 h at room temperature. Subsequently, the supernatant was added to fresh BioBeads (10 mg/70 μl) and shaking was continued for 10 min under the same conditions, yielding virosomes. The suspension was loaded atop a 5% sucrose (w/w), 145 mM NaCl, 2.5 mM HEPES, pH 7.4 layer in dialysis buffer and centrifuged for 90 min in a Kontron TST 60.4 rotor at $160\,000\times g$, 4°C onto a 40% sucrose cushion. Purified virosomes were collected from the interface.

3. Results

3.1. Effect of poly(ethylene-glycol) grafted lipids on influenza fusion with liposomes

Fusion of influenza virus with fluorescently labeled liposomes composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and gangliosides at a molar ratio of 6:3:1, with or without distearoyl-PEG-PE, was measured kinetically at pH 5.1, 37°C, by a resonance energy transfer method [24] as described in Section 2 (Fig. 1). Initial rates of fusion in the presence of 1 mol% liposomal PEG-5000-PE were more than half as low as those measured in the absence of PEG-PE, and the final extent of fusion was also affected, probably due to competition between fusion and low pH induced virus inactivation [27]. Free poly(ethylene-glycol) (MW 6000) did not affect fusion at concentrations equivalent to 10 mol% of the liposomal lipid (Fig. 1). Enhancement of influenza fusion by free poly(ethylene-glycol) has been described, but requires much higher concentrations [29]. No fusion was seen at pH 7.4 using either of these liposome preparations, in the absence or presence of free poly(ethylene-glycol) (not shown). PEG grafted lipids, especially those with short (myristoyl) acyl chains, have been shown to be more exchangeable between membranes than phospholipids, but the distearoyl-PEG-PE we used is stably associated with membranes [9,18]; it has been shown that no significant exchange takes place within 1 h of addition of an excess of neutral target liposomes [9]. All data reported in this paper are for shorter incubation times.

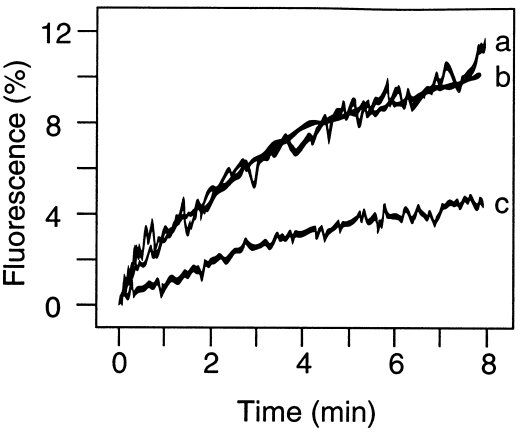

Fig. 1. Kinetic measurements of the fusion of influenza virus with liposomes, at pH 5.1, 37°C. The liposomes contained egg PC, egg PE, and gangliosides at a molar ratio of 6:3:1 (a and b) or 1 mol% PEG-5000-PE in addition to these lipids (c). Curve b was measured in the presence of 0.5 μM of free poly(ethylene-glycol), molecular weight 6000. Liposomal lipid concentrations were 5 μM, an equivalent amount of virus was added at time zero.

In order to determine the inhibitory effect of hydrophilic layers of varying thickness, the initial rate of fusion with liposomes containing a range of PEG-2000-PE or PE-5000-PE concentrations was determined. Inhibition was found to increase with increasing concentration and polymer molecular weight (Figs. 2 and 3). For PEG-2000-PE, half-maximal inhibition was found around 3.2 mol% of the PEG lipid, whereas for half-maximal inhibition with PEG-5000-PE, 0.8 mol% was required. Both of these concentrations are still in the 'mushroom' regime (see below). No fusion was found with liposomes containing 20 mol% PEG-2000-PE or 8 mol% PEG-5000-PE. Surprisingly, as is perhaps more clearly visible in the insets of Figs. 2 and 3, where the reciprocal fusion rate is plotted vs. the polymer concentration, the concentration dependence of the inhibition was not different for the 'brush' and the 'mushroom' conformations of the polymer. We estimate the transition between the two regimes to occur at around 1.6 mol% for PEG-5000-PE and around 4.9 mol% for PEG-2000-PE. These thresholds were calculated as outlined below.

The average distance between grafting sites D was first calculated as $D=(A/M)^{1/2}$, where A is the average molecular area of the lipids of the membrane, and M the mole fraction of the PEG grafted lipid [30]. Values of 60–70 $Å^2$, depending on acyl chain composition, were recently reported for the headgroup area of fully hydrated PCs, on the basis of com-

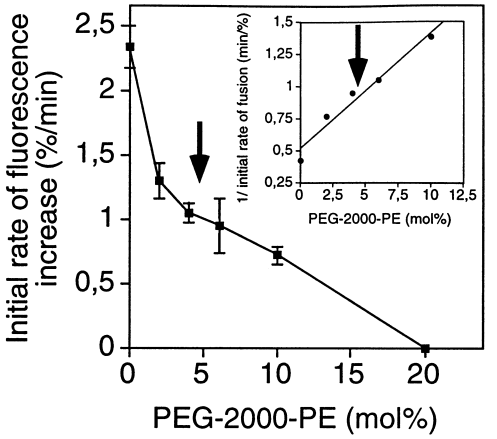

Fig. 2. Initial rate of fusion of influenza virus with liposomes containing PEG-2000-PE. The liposomes contained the indicated mol% of PEG-2000-PE in addition to PC, PE and gangliosides (molar ratio of 6:3:1). Other conditions as in Fig. 1. Initial rates of fusion were measured from the slopes of fusion curves such as those shown in Fig. 1. The data points are averages of at least two sets of data; error bars indicate ±1 S.D.; where no error bar is shown the S.D. is smaller than the drawn data symbol. The line between the data points is not a fit, but was just drawn to guide the eye. The inset shows the same data set, replotted as the reciprocal fusion rate vs. the concentration of PEG-2000-PE, and a linear fit of the data points (squared regression coefficient higher than 0.95). Arrows roughly indicate the transition between the 'brush' and the 'mushroom' concentration ranges.

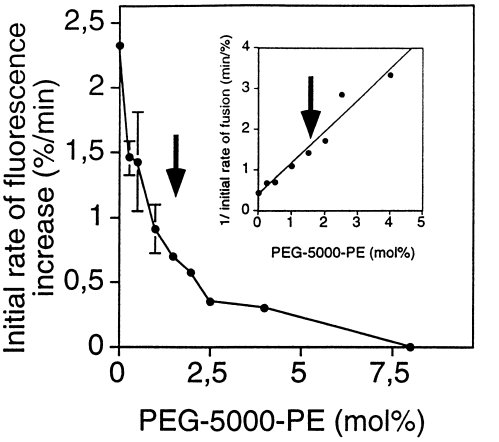

Fig. 3. Initial rate of fusion of influenza virus with liposomes containing PEG-5000-PE. Conditions as in Fig. 2, arrows indicate the brush/mushroom regime transition. The squared regression coefficient of the linear fit, replotting the reciprocal initial rate of fusion vs. the concentration of PEG-5000-PE, is higher than 0.95.

bined nuclear magnetic resonance data and X-ray diffraction measurements [31]. No such data are available for PE, but on the basis of its crystal structure, it has been suggested that in a fully hydrated bilayer, its headgroup should occupy the same area as the cross-section of its acyl chains, which is around 50 Å^2 in the liquid crystalline phase [32]. Monolayer compression data at bilayer equivalence pressures suggest an area of only 40 Å^2 for gangliosides if these are present in monolayers predominantly composed of PC [33]. Simply taking the weighed average of these data, we find a molecular area A of 55–61 Å^2 for membranes containing a 6:3:1 ratio of PC, PE, and gangliosides. According to de Gennes [13], the 'brush' regime applies if the size of one polymer coil in bulk solution, characterized by the Flory dimension R_f, is smaller than D, whereas for $D > R_f$, the 'mushroom' regime applies. The Flory dimension can be calculated according to $R_f = aN^{3/5}$, where N is the number of monomers per polymer, and a the size of one monomer (3.5 Å for ethylene glycol) [30]. Thus, for PEG-5000-PE we find that the 'brush' regime applies above 1.5–1.7 mol%, approximately, and for PEG-2000-PE, above 4.7–5.2 mol%.

3.2. Effect of poly(ethylene-glycol) grafted lipids on virus binding

Membrane fusion is preceded by binding of the virus to the target membrane, and the inhibitory effect of poly(ethylene-glycol) grafted lipids on fusion might therefore have been caused by steric inhibition of virus binding to the liposomes. Two different mechanisms contribute to the binding of virus to the liposomes used in the above experiments [26], which contain gangliosides in addition to zwitterionic phospholipids. The sialic acid residues of the gangliosides serve as receptors for the viral HA, binding the virus at neutral and at low pH. Moreover, after the low pH induced conformational change, insertion of the fusion peptide into the liposomal membrane contributes to the binding [6,27]. The latter mechanism also enables the virus, at low pH only, to bind to zwitterionic liposomes that do not contain receptors. In order to investigate the extent to which these two binding modes were affected by the presence of poly(ethylene-glycol) grafted lipids, liposomes where prepared containing PC and PE (2:1 ratio) containing PEG-2000-PE, and binding of the virus to these liposomes or liposomes containing gangliosides, as described above, was measured at pH 7.4 and 5.0 by a simple centrifugation assay (Fig. 4) [34]. As expected, in the absence or presence of PEG-2000-PE, the virus did not bind to liposomes lacking gangliosides at neutral pH. Ganglioside mediated binding at neutral pH was strongly affected by the presence

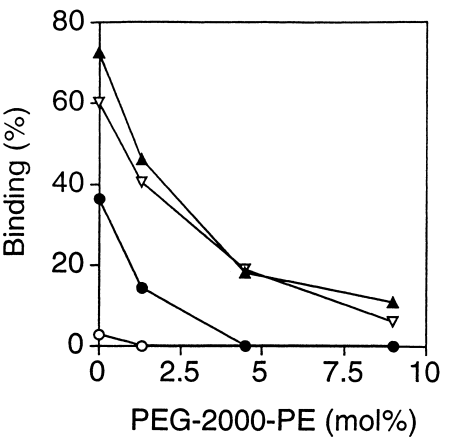

Fig. 4. Binding of influenza virus to liposomes. Virus was incubated with fluorescently labeled liposomes for 15 min at pH 5.1, 0°C (triangles) or pH 7.4, 0°C (circles), after which the mixture was neutralized, centrifuged, and the fluorescence of the liposomes copelleted with the virus measured as described in Section 2. Liposomal and viral lipid concentrations were 5 μM each. The liposomes contained the indicated amounts of PEG-2000-PE in addition to PC, PE and gangliosides (6:3:1 molar ratio, closed symbols) or in addition to PC and PE (2:1 molar ratio, open symbols). At this temperature and pH, the fusion that takes place within 15 min at 0°C is negligible.

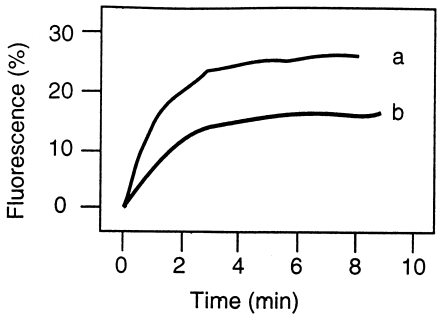

Fig. 5. Kinetic measurements of the fusion of reconstituted viral membranes with liposomes, at pH 5.1, 37°C. The reconstituted membranes contained viral lipids plus fluorescent probes (a), or additionally 3 mol% PEG-2000-PE (b); the liposomes contained egg phosphatidylcholine, egg phosphatidylethanolamine, and gangliosides at a molar ratio of 6:3:1. Liposomal lipid concentrations were 100 μM, viral lipid concentrations 5 μM.

of PEG-2000-PE in the liposomes, being completely abolished above 4.5 mol%. At pH 5.1, the presence of PEG-2000-PE reduced binding to ganglioside containing liposomes to about the level seen in the absence of gangliosides. Fusion peptide mediated binding was also affected, but only reduced to one-third of control at 4.5 mol% PEG-2000-PE. Given the relative distance of HA to the sialic acids of the gangliosides, in the distal part of the headgroup of these molecules, and to the hydrophobic interior of the target membrane, where interactions with the fusion peptide take place [6,7], these data are somewhat surprising.

3.3. Fusion of reconstituted viral membranes, containing poly(ethylene-glycol) grafted lipids, with liposomes

To determine how poly(ethylene-glycol) grafted lipids would affect fusion if they were present in the same membrane as the viral HA, reconstituted viral membranes containing these lipids were produced. Virus was solubilized with the detergent $C_{12}E_8$, viral membrane material purified, added to a dry film containing the fluorescent probes *N*-NBD-PE and *N*-Rh-PE or these probes plus PEG-2000-PE (3 mol% with respect to total membrane lipids), and then the membranes were reconstituted by removal of the detergent and purified by ultracentrifugation on a sucrose gradient as described in Section 2 [28]. The presence of PEG-2000-PE in the reconstituted membranes was confirmed by thin layer chromatography (not shown). As shown in Fig. 5, at pH 5.1, 37°C fusion of reconstituted membranes with or without PEG-2000-PE with a 20-fold excess of unlabeled liposomes (PC/PE/gangliosides 6:3:1 molar ratio) could be readily induced. In the presence of 3 mol% PEG-2000-PE, the initial rate of fusion was 40.4% of that in the absence of PEG lipid. No fusion was seen using either preparation at pH 7.4 (not shown). The pH threshold for fusion was around pH 5.6, identical for both preparations. These data indicate that, at this concentration, fusion is affected to about the same extent whether the poly(ethylene-glycol) grafted lipids are present in the HA containing membrane or in the target membrane.

4. Discussion

The data presented in this paper show that although poly(ethylene-glycol) grafted lipids inhibit influenza HA mediated membrane fusion whether they are present in the target membrane or in the reconstituted viral membrane, efficient fusion is still possible even when these polymers completely cover the membrane, forming a hydrophilic layer of several nanometers (below). The inhibitory effect is largely due to steric inhibition of HA-target membrane receptor binding and fusion peptide-target membrane interactions although other contributions such as bilayer stabilization by PEG-PE [35] cannot be excluded. Not surprisingly, inhibition is more extensive using lipids with a larger hydrophilic graft, and increases with increasing lipid concentration. The PEG-PE concentration dependence of inhibition was not clearly different with polymers in the 'brush' or in the 'mushroom' conformation.

In the low concentration 'mushroom' regime, the polymer grafts do not overlap, do not completely cover the membrane, and are mostly supposed to adopt random coil structures. The de Gennes theory predicts a maximum thickness $L = R_f$ for the hydrophilic layer covering the membrane, where R_f is the Flory distance (34 Å for PEG-5000-PE and 60 Å for PEG-2000-PE, see Section 3), considering the random coil structure of poly(ethylene-glycol) [13]. Needham et al. [36], considering that one end of the molecule is grafted, and that poly(ethylene-glycol) is a non-absorbing polymer whose local concentration near the membrane would be less than in the random coil conformation, have proposed that its maximal extension from the bilayer L be 7/5 R_f. For 4 mol% PEG-1900-PE in the 'mushroom' regime in a phospholipid/cholesterol membrane they thus calculated a value of 47 Å, in excellent agreement with the 50 Å they measured for these membranes by X-ray diffraction [14]. Thus, in the 'mushroom' regime, using their estimate, the maximal thickness of the hydrophilic polymer layer surrounding the membrane of the liposomes that we have used would be 48 Å for PEG-2000-PE, and 84 Å for PEG-5000-PE. However, assuming considerable flexibility in the conformation adopted by the graft, the *average* thickness would be much less, and increase with PEG-PE concentration. Also, potentially, in this regime, HA could still interact with parts of the membrane that are not covered by poly(ethylene-glycol) grafts.

That is no longer the case in the higher concentration 'brush' regime, where the overlapping polymers adopt an extended conformation, and the thickness of the hydrophilic layer surrounding the membrane could be considerably increased depending on the PEG-PE concentration. The variation of maximal layer thickness in this regime with polymer size and concentration, $L = aN(a/D)^{2/3}$, is well understood, and has, with some modifications, been confirmed experimentally [13,30]. For 2.5 mol% PEG-5000-PE, present in the target membrane, we thus find that $L \approx 70$–72 Å, and for 10 mol% PEG-2000 $L \approx 43$–44 Å. The initial rates of fusion observed at these concentrations were 15.0 and 42.5% of those in the absence of PEG lipids, respectively (Figs. 2 and 3). Therefore, efficient influenza fusion is thus observed with membranes completely covered with hydrophilic layers of several nanometers. Given the lack of a clear difference between the PEG-PE concentration dependence of inhibition in the 'brush' or the 'mushroom' regime on the one hand, and the fact that fusion is no longer observed at 9 mol% PEG-5000-PE ($L \approx 106$ Å), but also at 20 mol% PEG-2000 ($L \approx 55$ Å) on the other hand, we cannot give a more precise estimate. The lack of a difference between the 'brush' and the 'mushroom'

concentration ranges is probably due to the fact that the concentrations of PEG-PE that still allow fusion in the brush regime are at the low end of the brush concentration range, and that there is considerable overlap between L in this range and L in the mushroom range, especially if the latter could equal 7/5 R_f [36]. A smooth transition between the two regimes in this concentration range has also been reported using the surface force apparatus to measure the force between approaching PE membranes containing PEG-2000-PE [17] and a further subdivision of the mushroom regime into two regimes has been proposed [30].

Studies on the fusion between phosphatidylserine (PS) containing liposomes induced by Ca^{2+} have shown that fusion requires a close approach between the membranes; the Ca^{2+} bridges the two membranes, completely dehydrating the PS headgroups of the apposed membranes and thus overcoming the hydration repulsion which represents the main barrier to fusion (reviewed in [37]). In accordance with this hypothesis, PEG-2000-PE was found to sterically inhibit fusion of PS/PE (1:1) liposomes, resulting in more than half-maximal inhibition of fusion rates at PEG lipid concentrations of 0.5 mol% (in both membranes) [9]. Liposome fusion induced by the enzymatic conversion of zwitterionic lipids by phospholipase C was also readily inhibited in the presence of PEG-2000-PE, due to various mechanisms including steric inhibition of vesicle aggregation, fusion and inhibition of enzyme activity [10]. Some of these inhibitory effects started at 0.1 mol% PEG-2000-PE. Liposome fusion induced by free poly(ethylene-glycol) was also inhibited by the presence of PEG-2000-PE in the membranes, again because the grafted poly(ethylene-glycol) acted as a steric barrier [11]. Therefore, our data imply that either a close approach of membranes is not required for influenza fusion, or that in contrast to fusion induced between pure lipid membranes, the viral fusion protein HA acts in such a way as to overcome this steric barrier, allowing viral fusion with membranes covered with hydrophilic material. While the first possibility appears unlikely, the mechanism of the second remains to be investigated. The data presented in Fig. 4, showing that receptor mediated binding of virus to target membranes is more inhibited than fusion peptide mediated binding at the same concentration of PEG-2000-PE, could mean that penetration of the whole HA protein into the hydrophilic layer surrounding the target membrane, allowing HA-receptor interactions, is strongly inhibited for steric reasons. These data suggest that the more hydrophobic but much smaller fusion peptides would pass the hydrophilic poly(ethylene-glycol) layer to interact with the hydrophobic interior of the target membrane, perhaps propelled by the force of the conformational change in the protein.

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