Lateral Organization of Lipid Membranes Induced by Amphiphilic Polymer Inclusions

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Polymers belonging to a set of amphiphilic poly(acrylic acid) derivatives of varying hydrophobicity and charge density have recently been shown to slowly break small lipid vesicles and stabilize for hours or days transient mixed states such as membrane sheets, aggregates of mixed micelles, integral membrane proteins complexes, and so forth. We used giant unilamellar vesicles labeled with fluorescent probes to observe the evolution of a whole lipid-membrane including nondisruptive events, during the polymer-induced transition. The effect on the lipid bilayer depended strongly on the chemical structure and the concentration of polymer. Polymers of high hydrophobicity needed hours to disrupt the membranes. Before breakage, we observed intermediate states such as buds and filaments. Using less hydrophobic polymers, formation of flat domains was observed over hours at high polymer concentration (0.5 g/L). A single vesicle combined, over a few tens of micrometers, both curved fluorescent zones and flat zones of different compositions. More dilute conditions preserved the vesicle curvature and its low permeability to Dextran (M_w 9300 g/mol). The bound polymer/lipid ratio was on the order of 50−60 mg/g as measured by capillary electrophoresis analysis. These mixed systems give a unique experimental access to the effect of amphipatic macromolecules on membrane structure and properties. The slow kinetics of reorganization and generic formation of domains are specific features of the macromolecules in contrast with the well-documented effects of short surfactant molecules.

Introduction

In the conventional fluid mosaic model of the cellular membrane, lipids function mainly as a solvent for membrane proteins.[1] This view includes, however, the possibility of lipid organization and segregation in the membranes. Different lipid species often exhibit an asymmetric distribution between the two leaflets of a bilayer, essentially maintained by active "pumps" (e.g. flippase).[2] Recently, domains of submicronic size comprising cholesterol and sphingolipids (rafts) were isolated and considered as important "platforms" for the attachments of proteins, membrane trafficking, and signal transduction *in-cellulo*.[3,4] Several other models, although still a matter of debate, refer to subtle mechanisms for lipid segregation and their ability to function in biomembranes, for example, as a selective lipid concentration around a membrane protein (annulus assumption[5] or local reorganization in relation with hydrophobic mismatch[6]). Artificial systems such as giant unilamellar vesicles (GUVs) or lipid monolayers (notably Langmuir films) provide versatile tools to investigate membrane properties with a limited number of control parameters. Langmuir films containing two or more lipid species have been shown to self-organize in lipid domains upon variation of external conditions such as temperature, pH, ionic strength, and lateral pressure.[7] As regards the bilayers, small lipid domains in vesicles were visualized by electron microscopy[8] or two-photon fluorescence microscopy,[9,10] but there is a lack of experimental approaches allowing direct visualization of lipid domains in lipid vesicles. Bagatolli and Gratton have observed large "star domains" in mixed vesicles (DPPC/DPPE) that grow simultaneously on both inner and outer leaflets. The temperature-triggered separation between lipids was mapped using a two-photon imaging technique.[10] It is expected that the presence of nonlipid molecules such as membrane proteins can also significantly affect the formation of domains. The influence of inclusions of membrane proteins, of a cytoskeleton, or of an external polymer matrix is, however, difficult to investigate experimentally. We propose to use more versatile synthetic tools: amphiphilic polymers reversibly anchored in the bilayer of giant vesicles by *n*-alkyl side groups.

We[11] and other groups[12−14] seek to identify water soluble polymers that, upon binding to vesicles, markedly affect the shape, curvature, stiffness, or stability of the bilayer. Other studies of aqueous vesicle/polymer mixed systems

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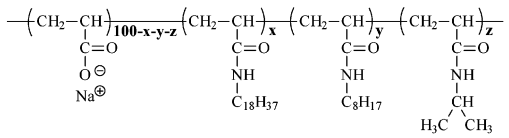
often aim at improving the stability and at controlling the permeability of liposomes for drug delivery or targeting, gene therapy, and so forth. Stabilization is usually obtained by loose hydrophobic anchoring of water soluble chains that does not significantly perturb the bilayer organization, such as alkyl-modified Dextran or Pullulan with a low degree of substitution,[15,16] long poly(ethylene glycol) capped with one or two lipid anchors per macromolecule, or poloxamers.[17,18] Like detergents, amphiphilic macromolecules can, however, strongly modulate permeability[14,19,20] and curvature energy[13] of membranes, solubilize lipids and form mixed micelles,[21] or hemolyze red blood cells.[22] Furthermore, polymer adsorption can enhance the fusion of membranes.[23,24] The mechanisms of these polymer-induced reorganizations of membranes are essentially conjectural, although it is clear that the hydrophobicity of the polymer plays an important role. We are interested in comparing the capacities of a set of amphiphilic macromolecules of varying hydrophobicity to induce local perturbations of the lipids. Various alkylamines were grafted onto poly(acrylic acid) precursors. Polymers of small modification rate were obtained by grafting octadecylamine onto a few mole percent of the carboxylic acid groups. Highly hydrophobic polymers were grafted with octylamine and isopropylamine up to a final density of 25−65 mol % of the monomers. The modified poly(acrylic acid)s adsorb onto unilamellar vesicles by simple mixing and markedly change the dispersion state of lipids. The more hydrophobic ones solubilize the membranes into mixed micelles.[11] This paper employs light-scattering measurements, capillary electrophoresis, and optical microscopy to determine how the inclusion of macromolecules into giant unilamellar vesicles affects the curvature and stability of the membrane and the spatial distribution of lipids. An important feature of these polymer/lipid systems lies in their very slow kinetics of evolution, allowing us to observe a succession of intermediate states during hours and sometimes days after the mixing of a polymer with vesicles.[11,25]

Materials and Methods

Polymer Preparation. The alkyl-modified poly(acrylic acid)s were derived by grafting alkylamines, at random along the chain of a poly(acrylic acid) precursor having a nominal molecular weight of either 5000 (Aldrich Chemical Co., Milwaukee, WI) or 150 000 (Polysciences Inc., Warrington, PA), using the procedure developed by Wang et al.[26] Gel permeation chromatography measurements performed in $NaNO_3$ solution gave a number average molecular weight of 6000 and 130 000, respectively, for these two polymers.[27] Although polymers with a wide range of degree of modification were synthesized, the experiments described in this paper are restricted to two distinct structures: (1) highly charged polymers carrying 3 mol % of C18 side groups and (2) highly grafted polymers having 25 mol % of C8 groups and possibly an additional 40 mol % of C3 (isopropyl) side groups (Figure 1). The different amphiphilic macromolecules are referred to as *N*-*x*C8-*y*C3 or *N*-*x*C18, with *N* their molecular weight in kilograms per mole and *x* and *y* the degree of grafting in mole percent (Figure 1). Aqueous polymer solutions (above 10 g/L) were prepared under magnetic stirring at least 18 h before use. They were diluted to the final range, 0.25−1 g/L, a couple of hours prior to use, by adding the buffer used for GUV preparation: 50 mM glucose, 10 mM bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane-HCl, pH 7.0 (BisTRIS Sigma Chemical Co., St. Louis, MO).



Name	Molar fraction of the monomers along the backbone			
	octadedyl (x)	**octyl (y)**	**isopropyl (z)**	**ungrafted (100-x-y-z)**
5-3C18	3 ± 0.3%	NG	NG	97 ± 0.3%
150-3C18	3 ± 0.3%	NG	NG	97 ± 0.3%
5-25C8	NG	25 ± 3%	NG	75 ± 3%
5-25C8-40C3	NG	25 ± 3%	40 ± 3%	35 ± 6%

Figure 1. Molecular structures of hydrophobically modified poly(acrylic acid)s. NG: no graft. The structure is deduced from ^{1}H NMR of a 1% polymer solution in D_2O.

Preparation of Giant Unilamellar Vesicles. Giant unilamellar vesicles (GUVs) were produced by an ac electric field controlled swelling of a lipid film, a method introduced by Angelova and Dimitrov.[28] We followed a similar procedure described by Mathivet et al. that essentially adds to the Angelova method formation in very high yield of unilamellar vesicles.[29] Note that, for cell membrane modeling, it is important to have a homogeneous population of unilamellar vesicles with a well-defined interior and a size comparable to the size of cells. Stock solutions of egg phosphatidylcholine (egg-PC) were made in a chloroform/methanol mixture 9:1 v/v at the lipid concentration 0.25 g/L. Egg L-α-phosphatidylcholine (egg-PC) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The fluorescent phospholipid 1-acyl-2-[12-(nitrobenz-2-oxa-1,3-diazol-4-yl)aminostearoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) was synthesized according to Colleau et al.[30] This lipid probe comprises a mixture of molecules that are similar to egg-PC lipids in terms of both headgroup and the natural variability of both length and degree of saturation of the "1-acyl" fatty chain. The fluorescent probe NBD-PC was added to the egg-PC stock solution at a final NBD-PC fraction of 5 mol % of the whole lipid content. The lipid solution was spread over the conducting sides of a chamber and vacuum-dried for 2 h (see Mathivet[29]). GUVs were formed in a "swelling solution" containing 50 mM saccharose and 10 mM BisTRIS/HCl buffer, pH 7. Vesicles were sucked from the chamber using a microsyringe and kept at 4 °C under argon. From previous experiments, it was estimated that the yield of lipid dispersion was about 40%. The final lipid concentration in the GUV solution was therefore on the order of

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0.01 g/L. For experiments on vesicle leakage, a rhodamine isothiocyanate Dextran (RITC-Dextran) was added in the "swelling solution" prior to vesicle formation (RITC-Dextran concentration 4 g/L, Dextran molecular weight 9300 g/mol with a rhodamine content of 0.004 mol/mol glucose, supplied by Sigma, St. Louis, MO).

Microscopy Experiments. Samples were observed by either phase contrast or epifluorescence in an inverted microscope (Zeiss IM 35). The fluorescence was excited by an argon laser (Innova 90-5, Coherent) at either wavelength 488 or 514 nm and at a maximum power/exposed area of 125 kW/m^2. Images were captured in a LH4036 video camera (LHESA, France). The vesicle preparation in the swelling solution (50 mM saccharose, 10 mM BisTRIS/HCl buffer, pH 7.0) was diluted 2-fold in a less dense solution comprised of 50 mM glucose, 10 mM BisTRIS/HCl buffer, pH 7.0. The dilution preserved the osmolarity and vesicle integrity while inducing the sedimentation of the vesicles toward the bottom of the observation cell and, if applicable, resulted in a decrease of RITC-Dextran concentration in the external solution as compared to the inner volumes of the vesicles. A 20 μL droplet of the diluted preparation was deposited on a glass cover slip previously glued with silicone to the bottom of a holed Petri dish, and subsequently covered with a second glass slip to limit evaporation. The distance between the top and bottom of the slips was 0.8 mm, making the isolated volume equal to 500 μL. Supplementation with polymer was achieved by adding 20 μL of a polymer solution (0.25−1 g/L in 50 mM glucose, 10 mM BisTRIS/HCl buffer, pH 7) a few minutes before recording of the first image. Reference samples were prepared by a similar 20 μL addition of a 50 mM glucose solution. When samples had to be incubated for hours, water was poured in the periphery of the Petri dishes, a cover was sealed with Parafilm M (American National Can, WI), and the samples were kept at 4 °C. We especially ensure that the osmolarity difference between the internal (saccharose) and external (glucose) solutions remains below a few milliosmolar to prevent shape changes due to osmotic pressure.

Association Isotherms by Capillary Electrophoresis. The fraction of polymer bound to the vesicles was measured in polymer/small unilamellar vesicle (SUV) mixtures instead of GUVs. Egg-PC SUVs were prepared by sonication using the following procedure: a film of lipids (egg-PC, Avanti Polar Lipids, USA) was layered on the side of a flask by evaporation to dryness of a chloroform solution at 50 °C under vacuum. The film was hydrated with the buffer solution (10 mM sodium phosphate/NaOH, pH 7.2) at 40−50 °C under nitrogen. The resulting suspension at about 3 g/L lipid was sonicated using a vibracell sonifier (Bioblock Scientific) at the power 600 W for 6 min, alternating 7 s bursts and 3 s rest periods. Finally, the sample was filtered through a 0.45 μm Millex filter and kept at rest for a couple of hours before being supplemented with polymers. The average hydrodynamic diameter of the vesicles was determined by dynamic light scattering to be 120 nm. It was found to be independent of the presence of the short polymers 5-3C18, 5-25C8, and 5-25C8-40C3 (see ref 11 for the description of the measurement). The size and polydispersity of the vesicle preparations were stable for at least 1 day. Although the unilamellarity was not controlled on these preparations, a similar procedure carried out on dipalmitoylphosphatidylcholine/dipalmitoylphosphatidic acid mixtures was shown to give essentially SUVs of about the same diameter until the lipid concentration was on the order of 10 mg/mL.[11]

In the mixtures, the amount of polymer bound can be determined by capillary electrophoresis. Experiments were carried out on a Beckman PACE-MDQ system fitted with a UV−visible diode array detector. A bare silica capillary of 31 cm × 75 μm was coated with poly(ethylene glycol) (PEG molar mass 400 000, SERVA New York) by successively flushing with water for 1 min, 0.1 M HNO_3 for 1 min, 0.1% PEG in water for 2 min, and the separation buffer for 2 min. The separation buffer was the same as the dispersion medium of the SUVs. Continuous injection analysis was initiated by immersing the inlet of the capillary in a sample and the outlet in the separation buffer. A positive voltage of 10 kV was applied at the outlet to achieve continuous injection of the polyanions and vesicle/polymer complexes in the capillary. According to their electrophoretic mobilities, the species were detected by two abrupt increases of the absorbance through the detection windows placed close to the outlet. Because of the continuous injection scheme, the free polymer formed the first large zone without modifying the adjacent next zone that corresponded to the mixture of SUV and polymer in equilibrium. This procedure enabled us to measure the concentration of free polymers without perturbing their equilibrium with SUVs (see refs 27 and 31 for further details). Data were essentially collected at fixed lipid concentration (1 g/L), increasing the polymer concentration. In the case of 5-25C8, additional measurements performed at 0.5 or 2 g/L lipid essentially gave the same results. After each run, the capillary was rinsed and recoated using the same sequence of solutions as described above.

Results

In the case of small unilamellar vesicles (SUVs), previous experiments performed at pH 7.0 with the same hydrophobically modified polyacrylates (HMPAs) have shown two extreme behaviors depending on the chemical structure of the polymer. Coating and long-term stabilization of the vesicles were observed with polymer having a low fraction of alkyl side chains (5-3C18 in Figure 1), whereas breakage and formation of mixed micelles resulted from the use of HMPAs with more than 25 mol % of octyl groups.[11] It is known that SUVs respond to stress by adjusting the membrane tension and eventually breaking.[32] The use of giant unilamellar vesicles (GUVs) enabled a more direct observation of intermediate structures formed upon mixing, adding also a possibility to experiment on the influence of the polymer adsorption on the shape and curvature of the membranes. To investigate the effect on bilayers of various HMPAs at varying concentration, stock solutions of GUVs were prepared before being supplemented with a polymer solution.

Morphological Effects of Polymer Interaction with Membranes. Figure 2 shows the result of adding HMPAs to GUVs at 4 °C (Figure 2b−d) in comparison to that for vesicles in the absence of polymer (Figure 2a). An aliquot of HMPAs (20 μL) was mixed with a drop (20 μL) of the vesicle solution to reach a final concentration of 0.125 g/L. This concentration (the lowest used in the study) corresponded to a large excess of polymer as compared to lipids (about 10 g/g, i.e., 14 molecules of octyl group per lipid, or three octadecyl groups per lipid). In the case of HMPAs having a high charge density (5-3C18, 150-3C18, and 5-25C8), the membranes that were floppy and fluctuating before polymer addition (see obloid shapes in Figure 2a) immediately turned to perfectly spherical and taut vesicles upon polymer addition (i.e. within less than the handling time of about 2 min). The membrane fluctuations remained completely frozen for hours, and we did not observe any modification of the shape and sizes of GUVs over a few days. Because it is possible to change the shape of a vesicle by either variation of the spontaneous curvature or by modification of the area/volume ratio, this effect essentially indicated that polymer binding immediately changes one of these parameters. In contrast to the cases of the remaining HMPAs, incubation with 5-25C8-40C3 destabilized the membranes. The GUVs did not exhibit a marked change in tautness, remaining floppy during the first couple of hours following the addition of 5-25C8-40C3. Small budding and filaments formed slowly. The latter are "hairy" structures rapidly fluctuating around isolated GUVs (Figure 2c). Images taken 3 days after mixing (Figure 2d) showed elongated structures,

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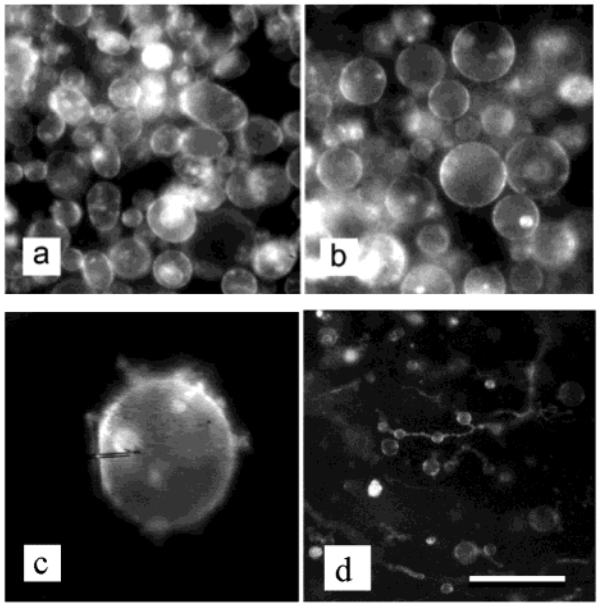

Figure 2. Images by fluorescence microscopy of GUVs in 50 mM glucose, 10 mM Bis-Tris/HCl buffer, pH 7.0, and with/without the presence of a polymer at a final concentration of 0.125 g/L: (a) reference floppy vesicles (16 h old, 4 °C) in the absence of polymer; (b) spherical vesicles obtained by a 16 h incubation at 4 °C in the presence of 5-3C18; (c) protruding filaments and budding observed after a 1 h incubation in the presence of 5-25C8-40C3 (The reticule on the left of the vesicle corresponds to 8.6 μm.); (d) loss of GUVs and formation of filaments after 3 days in the presence of 5-25C8-40C3. Fluorescent probe: 5 mol % NBD-PC. Photographs a, b, and d are at the same scale (scale bar in part d represents 50 μm).

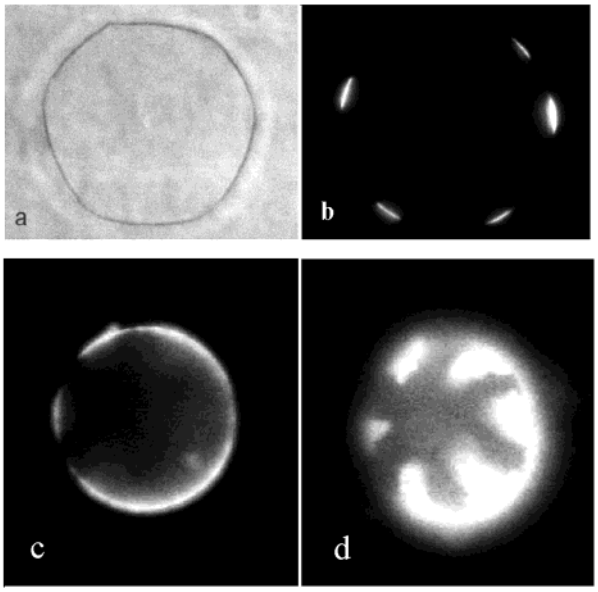

Figure 3. Images of GUVs kept for 1 h in the presence of 0.5 g/L 5-25C8 as observed by epifluorescence (excitation wavelength: 488 nm) (b−d) or phase contrast microscopy (a). A GUV observed with an objective having ×100 magnification and a sharp depth of field (a and b). Its periphery was essentially visible. Note that the nonfluorescent domains in part b correspond to the flatter parts in part a. Another GUV was observed with an objective having ×40 magnification and a higher depth of field, focusing either the vesicle periphery (c), like photograph b, or the upper crown of the vesicle (d). Photographs were subsequently rescaled to reach the same final magnification. The actual vesicle diameter was about 29 μm (a and b) or 32 μm (c and d).

types of small vesicles connected by highly fluctuating lines (their rapid movements made it difficult to focus them for photography), while the control incubated at similar conditions without polymer looked essentially like Figure 2a. The formation of small vesicles connected by filaments (necklaces) may share some similarity with the pearling instability of lipid vesicles, a well-documented transition[33] that was reported to occur upon addition of amphiphilic polymers.[12,25] In our experiments, however, the number of vesicles or elongated necklaces was found to be significantly lowered upon incubation as compared to the initial density of vesicles (compare parts a and d of Figure 2). A possible exchange of the internal saccharose content with external glucose, enabling vesicles to float everywhere in the drop instead of being brought down by their higher density, cannot explain the lack of objects because the pictures taken above the bottom plan only contained filaments and broken membrane sheets. The breakage of the vesicles upon addition of 5-25C8-40C3 may share some similarity with the solubilization in mixed lipid/polymer micelles as obtained with dipalmitoyl phosphatidylcholine SUVs.[11]

Upon 4-fold increase of the concentration of the nondisrupting polymers (5-3C18, 5-25C8, and 150-3C18), the results were essentially unchanged during the first hour: homogeneous and spherical GUVs without membrane fluctuations were immediately obtained. Longer incubation periods at 4 °C caused the formation of "dark spots" in the membrane. The spots slowly enlarged along the fluorescent periphery of the vesicles observed with a sharp depth of focus (Figure 3b and c). These dark domains retained the integrity of the membrane at the scale of 1 μm, as shown by the absence of discontinuities (and of holes) in the phase contrast image (Figure 3a). At the end of this growth, when the periphery has been almost completely invaded by the nonfluorescent zone, GUVs became polygons, as seen by phase contrast microscopy. It was apparent that the nonfluorescent parts were significantly flatter than the fluorescent ones. For instance, in the polygonal shaped GUVs, all the curved domains and edges corresponded to the few bright spots still visible by fluorescence (Figure 3b). With a larger depth of focus, a larger part of a vesicle was seen instead of a sharp section of its diameter. Under this condition, the dark domains were identified as facets that can invade a significant fraction of the vesicle's surface within a 1 day incubation (Figure 3d). As NBD-labeled egg-PC lipids were initially distributed homogeneously in the membrane, the heterogeneity of fluorescence pointed out that lateral heterogeneities were formed affecting the environment or distribution of the NBD-lipid. The almost complete disappearance of fluorescence indicates that all the major components among the mixture of lipid probes were equally affected by the phenomenon. In addition, both leaflets being initially labeled, these results showed a strong coupling between the development of the lateral heterogeneity on both sides of the membrane. Note that the development of these domains took place within a characteristic time markedly shorter than the typical flip-flop half-time of the lipids in unperturbed membranes (~10−15 h).

Permeability: Polymer Penetration and Vesicle Leakage. The ability of hydrophilic molecules or amphiphilic poly(acrylic acid) derivatives to pass through the membrane and equilibrate between the internal and external compartments was examined. First, experiments were conducted on leakage of RITC-labeled Dextran, a

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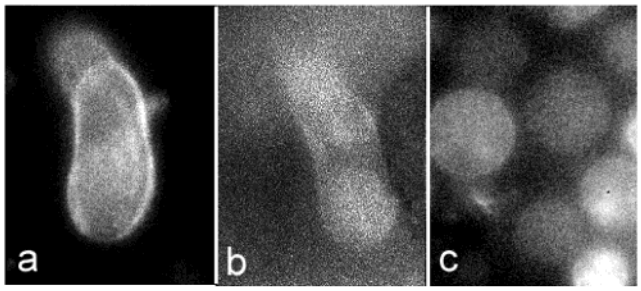

Figure 4. Images of GUVs with lumenal entrapped rhodamine-Dextran and incubated in the presence of polymer at 0.125 g/L. The vesicle membrane was labeled with 5 mol % NBD-PC. Polymer 5-25C8-40C3 and incubation for 1 h 45 min; observation of the membrane (NBD) using the excitation wavelength 488 nm (a) or of the rhodamine excited at 514 nm (b). After incubation with polymer 5-3C18 and incubation for 72 h, excitation wavelength 514 nm (c). N.B.: the fluorescence of the NBD-PC probe was faintly excited at 514 nm too.

soluble molecule of typical diameter 29 nm[34] initially trapped into the vesicles. The bilayers were labeled using NBD-PC. Excitation at 514 nm thus revealed the soluble probe, while the fluorescence excited at 488 nm delimited the vesicle boundary, and the presence, if any, of filaments or local distortions of the membrane. Figure 4a and b shows two images of the same sample observed at either wavelength after a 2 h incubation of the vesicles in the presence of the disrupting polymer 5-25C8-40C3. The diffuse fluorescence of RITC-Dextran in the whole image contrasted with the sharp and closed boundary corresponding to NBD-PC fluorescence in the bilayer, a result that was consistent with both an almost complete release of the Dextran and the preservation of the membrane's continuity at the scale of 1 μm. Because of the method of vesicle loading with Dextran (see Materials and Methods), a fraction of "free" Dextran was, however, present in the outer medium, even in the initial state before polymer addition. Despite the corresponding level of bulk rhodamine fluorescence, the contrast between the fluorescence of trapped Dextran and the outer medium was nevertheless significantly higher preceding the addition of 5-25C8-40C3 polymer (not shown). It compared well with the image in Figure 4c corresponding to the absence of Dextran release with the nondisruptive polymer 5-3C18. Remarkably, excellent lumenal retention of Dextran was found to last for days in the latter case (Figure 4c). While 5-25C8-40C3 rendered the vesicles permeable to the large hydrophilic Dextran, the less hydrophobic polymers 5-3C18 and 5-25C8 did not.

The localization of bound HMPAs was also investigated. Direct observation using a fluorescent polymer was difficult because of the low amount of bound HMPAs as compared to its bulk concentration (see paragraph below). Observations of the peculiar objects definable as "vesicles-in-vesicles" (VIVs) provided, however, information on the absence of penetration of HMPAs through the bilayer. A few among the largest giant vesicles contained by chance smaller vesicles that were trapped in their internal compartment. Upon addition of HMPA (5-25C8 or 5-3C18), the thermal undulations of the external membrane of these VIVs were immediately frozen and the outer shape became perfectly spherical (Figure 5), as for the single vesicles (Figure 2a and b). Brownian distortions of the small internal vesicles, however, continued for hours after addition of HMPAa (Figure 5). This behavior pointed

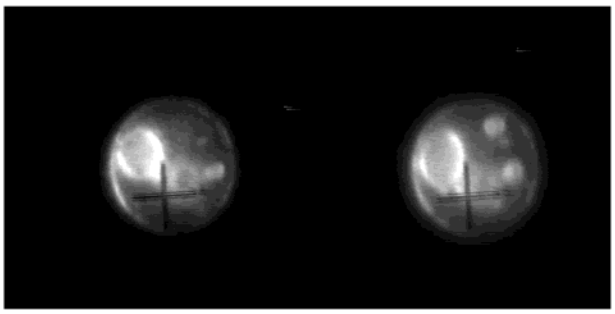

Figure 5. Photographs of a vesicle entrapped in a larger vesicle (VIV) at two different times separated by a few seconds. The vesicle solution has been supplemented with polymer 5-3C18 to a final concentration of 0.25 g/L. The images obtained a few minutes after supplementation were essentially similar to those shown here after an incubation for 5 days: the external bilayer appeared rather taut, while the shape of the internal vesicle (in the upper-left quarter of the reticule) fluctuated. The reticule size represents 8.6 μm.

clearly to the absence of HMPAs in the inner compartment of the largest vesicle of the VIV. The external membrane is found to be impermeable to HMPAs.

Adsorption Isotherms. As judged from the polymer/lipid weight ratio used, in the range 10−40 g/g, the effects described above were likely to develop in the presence of a large excess free HMPAs. We have quantified the exact amount of HMPA bound by implementing, with egg-PC SUVs, a capillary electrophoresis method often called frontal analysis or continuous injection mode (Materials and Methods). The vesicles (that carry charges when they associate with some polyanionic HMPAs) migrated in an electric field at pH 7.2 ($NaOH/NaH_2PO_4$ buffer, 10 mM) but slower than the free HMPAs. At conditions similar to those used with giant vesicle preparations, the free HMPAs were thus separated from a mixture of HMPAs and SUVs (see refs 27 and 35 for a detailed explanation of the principle). As reported with other colloid/polymer associations,[27,35] the method does not perturb the equilibrium between free and bound species. From measurements performed at varying polymer/lipid ratio, the association isotherms were calculated (Figure 6). A saturation of the bound polymer/lipid ratio was reached at about 0.05 g/g with 5-25C8 or 0.065 g/g with 5-25C8-40C3. The difference between both polymers remained close to the experimental error (relative errors larger than 10% close to the saturation). In terms of molar fraction, the saturation plateaus correspond to about 0.09 octyl group bound per lipid. The composition of coated vesicles reached this saturation plateau above a concentration of unbound polymer of about 0.1 g/L, corresponding to an excess of free polymer as compared to bound polymer (0.05 g/L), despite the high concentration of lipids (1 g/L). In the case of 5-3C18, however, the high mobility of vesicles, beyond a free-polymer concentration of 0.06 g/L, made it difficult to separate free polymer and coated vesicles, hampering the collection of reliable data above the concentration sufficient to saturate the vesicle in the latter cases. As expected for a more hydrophilic and highly charged polymer, the beginning of the isotherm of 5-3C18, however, seemed to correspond to a lower association as compared to the case of 5-25C8.

Discussion

The association of HMPAs with membranes modifies the properties of GUVs in very different ways depending

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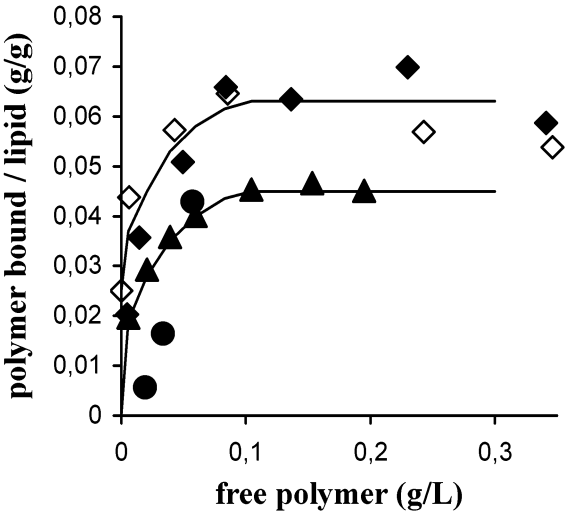

Figure 6. Adsorption isotherms of the short polymers onto egg-PC SUVs. Results from capillary electrophoresis analysis of mixtures containing a polymer and egg-PC SUVs, in 10 mM NaH_2PO_4/NaOH buffer, pH 7.2: (▲) polymer 5-25C8; (◆) 5-25C8-40C3; (◇) 5-25C8-40C3 reanalyzed after a 24 h incubation time; (●) 5-3C18. Lines are to guide the eye.

on the polymer chemical structure and the concentration. As expected for molecules that solely bind to the outer layer of the membrane, the presence of nondisruptive polymers made the GUVs more spherical and more stable. The increase in the number of molecules in the external leaflet, together with highly likely steric and electrostatic hindrances between bound macromolecules, could explain these observations. Beyond the well-documented coating of liposomes, long incubation time resulted in lateral heterogeneities and flatness. The binding of highly hydrophobic HMPAs on GUVs membranes was found to result in a huge variety of shapes including protrusion of filaments, and transient necklaces of vesicles. Since the formation of tubes or beads was reported in other GUV/polymer systems[12,13] and generic mechanisms proposed for the shape transition and their propagation rate,[25] we focus the discussion on formation of facets and membrane breakage. In terms of structural parameters of the macromolecules, the density of hydrophobic side groups along the polymer backbone together with the charge density was found to be critical for the influence of polymer on the bilayer. A clear difference exists between the behavior of either 5-25C8 (domain formation, retention of Dextran) or 5-25C8-40C3 (permeability preceding the membrane disruption), although those macromolecules faintly differ in terms of hydrophobicity. Because a difference in the chemical structure implies a change of both the hydrophobicity and the density of anionic units along the polymer, it is important to note that a change in the charge density alone modifies the capacity of the HMPAs to break the bilayers. At pH 7.0, the polymer is partially ionized; full ionization of the carboxylic groups was found to require a pH above 8.0 (not shown). At pH 7.2, none of the latter HMPAs were able to break SUVs as they did at pH 7.0 (not shown; experiments partially described in ref 11). Thomas and Tirrell have also demonstrated the high sensitivity of comparable systems (mixtures of poly(2-ethylacrylic) acid and various vesicle dispersions) to small modifications in polymer charge density as modulated by pH shifts.[21,22] Similarly, membrane permeabilization by poly(2-ethylacrylic) acid and transient pore opening have been shown to occur close to the critical conditions that break the vesicles.[14] These phenomena were correlated with the critical effect of ionization on both the extension of poly(2-ethylacrylic) acid and the polymer solubility in water. Because pH 7.0 was not far from the pH below which our HMPAs were insoluble (pH 6.5), a similar importance of ionization can be tentatively extended to HMPAs though complementary investigations on the pH-dependent behavior of these polymers would be required. The major difference from Tirrell's work was that all the HMPAs considered were hydrophobic enough to bind to the membrane at any pH. Both a low charge density and the presence of hydrophobic groups are crucial for the breakage of membranes.

With regard to nondisruptive events, formation of dark domains appears less sensitive to the ionization and the type of hydrophobic side group because it was observed with both 5-3C18 and 5-25C8. It seems that, in the absence of membrane rupture, lateral heterogeneity of a few microns in dimension requires only enough time to develop very slowly, over hours. The flatness of the lateral "dark" domains indicates a local increase in stiffness as compared to that of the curved parts of the GUVs. The adsorbed polymer layer may apply a stress to the membrane due to interpolymer repulsion, or self-organization forming an external elastic network bound to the lipids.[36] The polymers were, however, found to remain only on the outer leaflet which was likely to occur with HMPAs carrying 97 mol % of anionic groups (5-3C18). Polymer adsorption on one side of the membrane together with the concomitant enhancement of surface charge would favor curvature rather than flatness because the increase in repulsion localized in small domains on the outer layer would be relaxed by an increase in local curvature, or budding. Stiffness as a result of the presence of anchored polymers repelling each other requires that the HMPAs can pass through the bilayer and coat the inner leaflet too. The vanishing fluorescence in the facets reveals that the flat domains exhibit a significantly different environment for lipids or a different composition. Lipid segregation and faceting has been observed in other GUV systems upon cooling binary mixtures of lipids, very likely as a consequence of a phase transition of one lipid[10] or specific mixtures of lipids.[9] The separation of a gel phase in unsupported bilayers of lipids was proposed as a reason for the formation of domains. Complex mixtures of lipids such as natural egg-PC do not exhibit a phase transition, and membranes are known to remain fluid even at the temperature 0 °C. Moreover, the lipid probe used in this study was not a single species molecule but a collection of NBD-labeled lipids differing in both length and degree of saturation of the "1-acyl" fatty chain. Therefore, the vanishing fluorescence of the probe means that the phenomenon was not sensitive to variations of the first fatty chain. The origin of lateral segregation of lipids by phase separation is therefore not obvious. We state, nevertheless, that the phase separation of a major component in egg-PC forming nonfluorescent rigid patches can explain our observations. Other tentative explanations of domain formation are listed as discussed below.

The decrease of fluorescence could be more specific to NBD/polymer interaction. For instance, it may result from a quenching by the adsorbed polymer, self-quenching of the probe, or specific extraction/expulsion of the probe. The absence of aromatic or halide quenchers in the polymer structure makes quenching highly unlikely. Some quenching would also occur at low polymer concentration, but

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the fluorescence did not markedly decrease upon addition of polymer at low concentration (0.125 g/L). It must also be emphasized here that the fluorescent group (NBD) in the lipid probe was grafted neither to the polar head nor at the end of the fatty chains. The latter two structures are known to promote the residence of the rather polar NBD group close to the polar interfaces of the bilayer and, consequently, close to the bound polymer. In the home-made probe, the location of the NBD group at the surface is, in principle, less probable because of its grafting on the C_{12} carbon of an octadecanoyl group (see Materials and Methods). In addition, the complete darkness indicated the absence of fluorescence on both sides of the membrane while the observation of VIVs showed that the polymers (5-25C8 or 5-3C18) do not enter in the vesicle. Even though a strong affinity of the NBD-lipid for the polymer would exist, the influence on the inner leaflet would require flip-flop of the probe. In unperturbed membranes, the typical flip-flop time of 10−15 h is markedly longer than the time for enlargement of dark domains. We can exclude self-quenching of the NBD-lipid as a reason for the origin of vanishing fluorescence. As an example, both the 80:20 and 50:50 mixtures of egg-PC and NBD-lipid were highly fluorescent under the microscope although self-quenching decreased their fluorescence down to the intensities of 83:17 and 95:5 mixtures, respectively. A local enrichment of NBD-PC by a factor markedly larger than 10 would be required to quench fluorescence. However, it is impossible that segregation from the initial 95:5 mixture can form NBD-PC domains so large that they invade significantly more than 5−10% of the whole vesicle surface. Another effect of the presence of polymer could be the selective solubilization of some lipids, especially NBD-lipids. The phenomenon would result in a marked modification of the GUV composition and possibly enrichment of the membrane with lipids having a high gel−fluid transition temperature. Though lipid solubilization in HMPA solutions was observed in similar systems,[11,21,37] the mixed micelles were only formed at conditions that broke the membranes. The 5-3C18 polymer did not change the size of SUVs in similar conditions,[11] making the solubilization of a significant fraction of lipids very unlikely.

Altogether, the preceding arguments favor the expulsion of NBD-lipids from both leaflets of the dark domains. A local phase transition, for example, the cocrystallization of part of the lipids and polymer bound, can induce the corresponding fractionation. The reason for the requirement of the presence of HMPAs at high concentration in the bulk, however, remains unclear. Because the HMPAs are polyelectrolytes, increasing their concentration from 0.125 g/L (no domains) to 0.5 g/L increases the ion concentration. A possible role of the ionic strength and osmotic pressure was, however, ruled out by the absence of domain formation in mixtures that were supplemented with 5 mM sodium chloride and 0.125 g/L polymer (both 5-3C18 and 5-25C8). As compared to samples containing 0.5 g/L polymer, the ionic concentration was essentially the same in the latter mixtures and the osmolarity was dominated by the glucose concentration in both mixtures. Owing to the large excess of polymer in the samples (up to 40 g of polymer/g of lipid, well above the 0.06 g/g of polymer bound/lipid on SUVs), the increase in the polymer concentration may, however, speed up the polymer adsorption. The alkyl side groups along the HMPAs are not expected by themselves to undergo a phase transition: comparatively, saturated lipids do not form a gel phase in the bilayer at temperatures above 0 °C. Moreover, the absence of marked differences between C8- and C18-modified polymers cannot be consistent with the great importance of the structure of the hydrophobic tails in a phase separation. We propose that the formation of a domain is essentially due to a co-organization of HMPA and lipids at the surface of the GUVs. The presence of polymer could change the solvation of the polar heads of the lipids, resulting in the modification of their self-organization. Further experiments are, however, required to validate this suggestion, aiming, for instance, at the quantitation of polymer adsorbed in the dark domains and analyzing the bulk concentration of lipids. The variety of fatty chains that compose the hydrophobic tails in egg-PC does not rule out the presence of a dominant compound: the fatty acid residue R1 is in most cases a palmitate (16:0, about 60 mol %) or a stearate (18:0, about 30 mol %); the fatty acid residue R2 is essentially the oleate (18:1, 66 mol %) or 18:2 (34 mol %) [Avanti Polar Lipids]. About one-third of the lipids are thus comprised of one 18:0 chain and one 18:1 chain, a compound that in chemically pure bilayers undergoes the fluid−gel transition at 6 °C. The surface fraction of dark domains deduced from the microscopy images seems to be consistent with the natural abundance of this major lipid compound (Figure 3c and d; it is worthy of note here that the image in defocused conditions represents only the upper crown of the vesicle). Nondisruptive HMPAs could be considered from that point of view, as a perturbation that may locally trigger selective lipid enrichment. Though triggered from the external leaflet, the phenomenon must be coupled with the internal leaflet in order to match with the observations. Additional investigations are required to validate the possible influence of HMPAs on lipid−gel transition in bilayers that contain several lipid species. Still, the determination of the temperature of phase transition was considered by Schroeder and Tirrell in mixtures of small vesicles comprising a single lipid and polymer. They found the occurrence of a second melting peak in dipalmitoyl phosphatidylcholine vesicles/poly(2-ethylacrylic) acid, 1.5 °C above the unaffected melting of pure DPPC, although the presence of two successive phase transitions was limited to a very narrow range of pH using a polymer that rapidly breaks the membranes.[38]

Conclusions

Synthetic polymers of adjustable structure combined with giant unilamellar vesicles provide a versatile tool to investigate the effect of macromolecule binding on the stability, permeability, and stiffness of biomimetic membranes. Though all the amphiphilic polymers tested in this study adsorbed onto the external leaflet of the GUVs, only the more hydrophobic ones subsequently permeabilized and finally broke the membrane. This agrees well with the existence of a critical hydrophobicity for rupture as proposed by Tirrell et al. On the other hand, the binding of essentially hydrophilic macromolecules exerts a strong influence on both the mechanical properties of the membranes and the growth of a heterogeneous environment of lipids in egg-PC bilayers. We found a rather small amount of bound alkyl side group of HMPAs as compared to lipids (∼0.1 mol/mol). The sensitivity of the polymer/lipid assembly to the polymer concentration and very slow kinetics of both domain formation and membrane rupture will merit further study on the origin of rate-limiting steps.

Other direct observations of domains and segregation in nonsupported bilayers exist, although not with the

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complete egg-PC mixture. Inclusions of macromolecules in the external leaflet may play the role of a segregating agent that locally modifies the lipid composition of the membrane, helping conventional phase separation to occur locally. Polymers having different hydrophobic anchors and different charge densities did not significantly differ in their capacity to induce domains. This seems to indicate that domain formation is a general phenomenon requiring essentially the adsorption of amphipatic and inter-repelling macromolecules in bilayers. Even though a direct comparison with the influence of extrinsic membrane proteins or the polymeric corona of natural membranes is certainly premature, such synthetic systems offer a powerful opportunity to show how a soft outer layer can profoundly affect the lipid organization. The structure of the macromolecules constitutive of the adsorbed layer could easily be modulated by simple chemistry. This opens interesting opportunities to explore the role of side groups of different structures and polymers of different dimensions, especially by varying hydrophobicity, charge density, and polymer length.

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