Bilayer vesicles and liposomes as interface agents

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Vesicles as interface agents flocculate or stabilize particulates, bacteria or mammalian cells. Synthetic cationic vesicles are antimicrobial agents killing bacteria and fungi at concentrations that barely affect cultured mammalian cells. Silica or latex become functional from coverage with bilayer-forming amphiphiles or phospholipids. Lipid self-assembly on particles allows receptor insertion and amplification of receptor–ligand recognition, *e.g.*, model pair monosialoganglioside GM1 and its ligand, the cholera toxin. Utility of self-assembled vesicles, bilayers or monolayers at interfaces is limited only by our own imagination.

1 Background

The physical and chemical factors which determine the deposition of bilayer vesicles from aqueous media onto solid surfaces are still poorly understood despite its importance (reference 1 and references therein).

In the eighties, liposome adsorption on clays, asbestos, Biobeads, gel filtration columns and membrane filters was incidentally observed and reported (reference 2 and references therein). Vesicle deposition onto a solid surface would be determined initially by the classical combination of a repulsive force arising from the interaction of the electrical double layers associated with the vesicle and the solid surface and the attractive dispersion force between the vesicle and the solid. Vesicles are not, however, permanent rigid structures, and depending on their size and chemical composition and that of the aqueous medium they can distort, aggregate, disrupt and fuse with each other. Deposition of vesicles onto a solid surface could give rise to any particular one or a combination of these processes. Unilamellar phosphatidylcholine vesicles were reported to break open and adhere to a mica surface to form a bilayer coating, in spite of the evidence for this being indirect as obtained from the measured separation between two surfaces when pushed together.[2] Further compression of the closely apposed bilayers may result in fusion into a single bilayer depending on the magnitude of short-ranged interaction forces.

Dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol (PI) from vesicles adsorb onto negatively charged Ballotini glass beads as a monolayer with their head groups uppermost.[2] Phospholipid monolayers with lipid haptens inserted are supported by hydrophobic glass and are useful for specific adherence of macrophages and cell surface recognition studies, but cannot serve as hosts for transmembrane proteins.[3]

Over the last decade, much effort was dedicated to preparing supported planar bilayers (SPB) adsorbed to planar hydrophilic supports such as glass, quartz, oxidized silicon and mica from the perspective of designing biosensors based on, *e.g.*, conducting properties of transmembrane proteins, receptor–ligand recognition, protein binding to lipid ligands, cell–cell recognition in the immune system (reference 4 and references therein). Essentially, two methods have been used to prepare such SPBs: (1) consecutive transfer of two lipid monolayers onto the surface *via* Langmuir–Blodgett techniques; (2) vesicle spreading and fusion onto the surface, *e.g.* fusion induced by traces of Ca^{2+}. However, interaction forces between vesicle and surface do not always determine bilayer deposition. Simple vesicle adhesion to the surface may also occur mainly when 'bulky' and/or strongly hydrated moieties in the lipid headgroup and/or on the surface generate a strong steric and/or 'hydration' repulsion, respectively.[1] For example, Nollert *et al.* found that vesicles formed from *Escherichia coli* lipids, a lipid mixture rich in lipopolysaccharides with bulky and strongly hydrated polarhead groups, did not form a SBP on glass, vesicles simply adhering and forming a supported vesicle layer.[4] Consistently, Tápias *et al.* also found adhesion of a vesicle layer of dioctadecyldimethylammonium bromide (DODAB), a lipid with a poorly hydrated polar headgroup, onto the rough and highly hydrated surface of *E. coli* cells, the electrostatically driven attraction between these cationic vesicles and the negatively charged cell surface driving the vesicle layer deposition.[5]

A central problem has been the structure of membranes and membrane proteins, one of the most exciting subjects of research in biology. When such membranes, either artificially made or purified, are placed on a solid support, such as mica or glass cover slips, imaging using the atomic force microscope (AFM) allows a high resolution, a clear advantage since membrane proteins cannot be resolved on cells.[6] Supported membranes on mica are stable under the AFM for repeated scans and in various buffers, a very important capability for

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biological applications that require full hydration for retention of the native structures. When a membrane of appropriate composition is made on a mica surface, peripheral membrane proteins can be easily added to the buffer to allow binding to the membrane. The most straightforward example is the case of the cholera toxin bound to supported bilayers that contain the cholera toxin receptor, the monosialoganglioside glycolipid GM1.[6–8] Shao and Yang have found that the stability of the toxin on fluid phase bilayers, such as egg-PC can be as good as that on gel phase bilayers, such as DPPC.[6] The success of AFM imaging this toxin at intermediate ionic strength (up to 150 mM) opened the real possibility of imaging reconstituted membrane proteins under physiological conditions.[6] Another significant example, is the reconstitution of gramicidin A incorporated in such supported bilayers.[9] Gramicidin A is a short, transmembrane peptide with channel function, consistently resolved as a channel-like depression of 1–2 nm.[9]

For integral membrane proteins, reconstitution into planar membranes has not been demonstrated. Main methods to incorporate the proteins into a supported planar membrane utilize the fusion of vesicles: either directly fusing vesicles that contain integral membrane proteins onto a supported substrate such as a piece of quartz or glass coverslip[10] or fusing them onto a substrate which was previously coated with a monolayer of lipids.[11] The mechanism of such events is not understood. Also, vesicles submitted to an osmotic shock directly spread at the air–water interface, with such a spread thought to result in a monolayer at the air–water interface with the integral membrane proteins incorporated.[12] Such a monolayer could then be picked up by a substrate with a preformed monolayer.[12] For those proteins with large masses protruding on both sides of the membrane, it is not clear how this spreading method could be effective. Possibly, progress in artificially accommodating these biomacromolecules will be achieved from biomimetic reconstruction of intermolecular interactions between lipids and highly hydrated and water soluble biopolymers such as biopolysaccharides.[13,14] These macromolecules will possibly offer adequate microenvironments, a sort of 'hydrated cushion', for bulky extramembrane moieties of transmembrane proteins to lie on. The initial conditions of the polymer layer were demonstrated to be a critical factor for the successful formation of a continuous bilayer atop a hydrated polymer layer.[15] The presence of the polymer cushion significantly alters the interaction potential. These polymer-supported bilayers could serve as model systems for the study of transmembrane proteins under conditions more closely mimicking real cellular membrane environments. Fig. 1 shows a latex particle with a double coating of a biopolysaccharide and a bilayer. In this case, the latex is negatively charged and was firstly coated with a positively charged chitosan layer and secondly, with a dioctadecyldimethylammonium bromide bilayer. The dark ring surrounding the particle is the DODAB bilayer which was positively stained before being imaged using transmission electron microscopy. The positive, dark stain surrounding the particle is due to adsorption of the molybdate anion at positive sites on the bilayer. The heavy element, molybdenum, strongly deflects the electron beam of the microscope generating the observed contrast and imaging of the dark ring.

On the other hand, the modification of metallic surfaces as ultrathin coatings of metals such as gold and silver has been a very active research area over the last two decades due to its great utility for technological applications such as protection of microelectronics and design of biosensors with optical or electrical detection. These ultrathin coatings of metals could be obtained by the chemisorption of alkane thiols and disulfides. Supported hybrid bilayer membranes (SBM) composed of a monolayer of phospholipid and a monolayer of alkanethiol associated with a thin gold film on glass have been useful as model lipid membranes for studying membrane receptor–ligand events either *via* surface plasmon resonance (SPR) (reference 16 and references therein) or impedance analysis (reference 17 and references therein) or *via* a combination of impedance analysis and SPR. SBM were used for studying membrane receptor–ligand and cell–cell binding events by SPR under conditions of continuous flow.[16] This kind of experiment should allow determination of equilibrium binding and kinetic rate constants between receptors and ligands.[16] The thin gold coating is optically transparent and light coupled into it generates plasmons that are either reflected or refracted at the interface. The angle of reflectance minimum is a function of the refractive index at the interface, so that the angle at which incident light will be in resonance with the surface plasmons is sensitive to the refractive index of the medium near a metal surface. The binding of ligands to surface-immobilized receptors alters the interfacial refractive index, allowing one to directly monitor a change in the molecular composition of the interface by monitoring the change in angle of the reflectivity minimum. The binding specificity to the bilayer was conferred by using POPC vesicles containing a biotinylated phospholipid for the preparation of HBM so that the SPR response as a function of time could be monitored upon injection of neutravidin (specific ligand) or bovin serum albumin (non-specific ligand).[16] The response to the specific ligand was approximately a threefold increase in signal, whereas the increase in signal due to BSA was negligible.[16] The versatility of the SPR approach was recently reviewed by Anne Plant.[18]

Whereas the vast majority of the literature focused on SPBs, supported spherical bilayers, SSBs, remained much less explored (reference 1 and references therein) despite their advantages over SPBs. Because particles are used as supports,

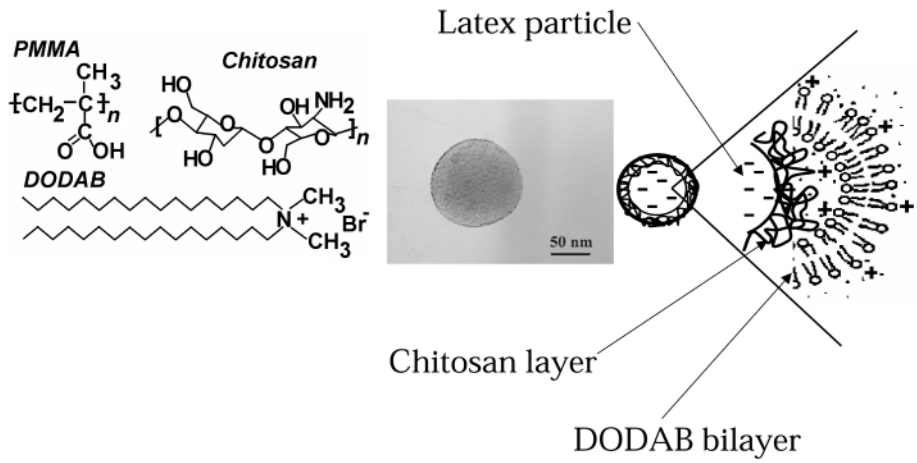

Fig. 1 Bilayer coverage over a 'water cushion' of an hydrated biopolymer. The bilayer is composed of DODAB and the biopolymer of a biopolysaccharide, chitosan, which is weakly charged at the pH of water. The micrograph shows the bilayer as a dark ring surrounding the particle.

SSBs offer a much larger total surface area than SPBs. Consequently, they allow quantitative analysis of lipid deposition using simple techniques such as determination of adsorption isotherms, electrophoretic mobility as a function of added lipid and usual particle sizing techniques such as light scattering for determination of the increase in mean particle size due to lipid deposition.[19,20] Moreover, they resemble the liposomes and vesicles in their invasiveness of biological microenvironments, potentially fulfilling as many applications in biology and medicine as the liposomes do: SSB might also deliver drugs, genes and vaccines (reference 1 and references therein).

2 Model surfaces and model vesicles

Fig. 2(a) illustrates some of the model surfaces that have been used for studies of the vesicle–surface interactions. Among the mineral surfaces the most commonly used are those from mica, glass, Si/SiO_2 wafers and silica. Among the polymeric surfaces, one of the most interesting is the surface available from polystyrene microspheres, which is composed by homodisperse polystyrene lattices dispersed in water. Among the biological surfaces, certainly one of the most important, and most intricate, is the cell surface. We have been dealing with the complex interaction between cell surfaces and vesicles from a physicochemical point of view since 1994.[5,21,22] These studies with cell surfaces revealed an unexpected finding: cationic DODAB vesicles can act as potent bactericides against several pathogenic bacteria of clinical importance (reference 21 and references therein).

Fig. 2(b) schematically shows the phospholipidic or synthetic bilayers that may enclose a water compartment to form the bilayer vesicle. Some lipids used to form vesicles are either synthetic amphiphiles such as dioctadecyldimethylammonium chloride (DODAC) or bromide (DODAB) or, sodium dihexadecylphosphate (DHP) (reference 23 and references therein), or natural lipids such as egg phosphatidylcholine (PC) or dipalmitoylphosphatidylcholine (DPPC). In the physical sense, vesicles are thermodynamically unstable because the symmetric membrane is curved and the excess energy of each vesicle due to its curvature is $8\pi K$, where K is the elastic bending modulus of the membrane (reference 23 and references therein). Vesicles are formed spontaneously only in the case of bilayers with very low values of K (reference 24 and references therein). A vesicle, however, is a much more stable physical entity than a micelle since the residence lifetime of one single molecule in the vesicle and in the micelle are *ca.* 10^4 and 10^{-4} s, respectively.[25] This explains why micelles or microemulsion droplets quickly disintegrate upon dilution whereas vesicles and liposomes made from phospholipids or double-chained synthetic amphiphiles (with very low values of critical micelle concentration) remain stable against dilution. Further reading about vesicles is available from the literature (references 23–25, and references therein).

3 Coverage of polystyrene lattices, silica or bacterial cell surfaces with lipid layers

Polystyrene microspheres interact with oppositely charged vesicles so that flocculation or stabilization of the particle dispersion takes place in accordance with the proportion of total surface area for vesicles and particles.[19,20,26] In Fig. 3, a mixture of cationic vesicles and anionic particles of similar sizes is taken as an example. Taking the particle and vesicle number densities as N_p and N_v , respectively, there are a number of situations that may arise depending on the N_v/N_p ratio. At $N_v/N_p < 1$, bilayer coverage generates a positively charged particle that may further attract the remaining negatively charged particles

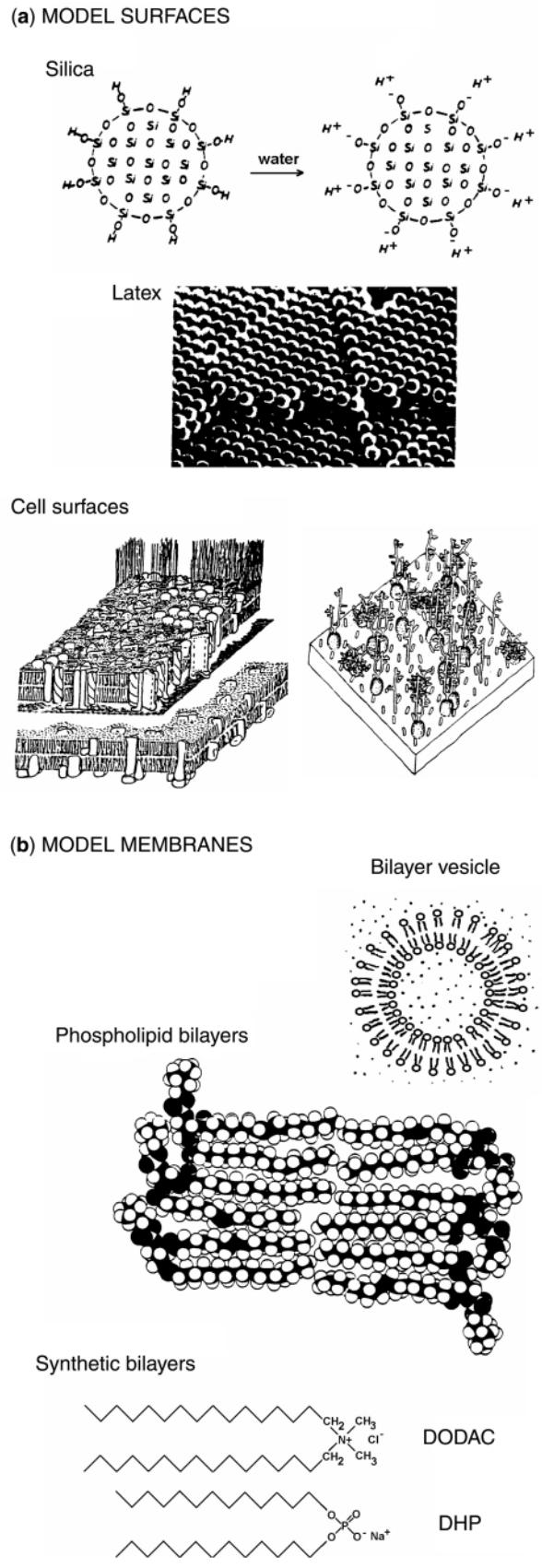

Fig. 2 (a) Some models of inorganic, organic and biological surfaces such as silica, latex and cell surfaces, respectively. (b) Some models for membranes such as closed unilamellar vesicles composed of phospholipids or of the synthetic amphiphiles dioctadecyldimethylammonium chloride (DODAC), cationic, or sodium dihexadecylphosphate (DHP), anionic.

generating an aggregate and thereby increasing the mean *z*-average diameter of the mixture. At $N_v/N_p > 1$, bilayer coverage and the consequent electrostatic repulsion causes

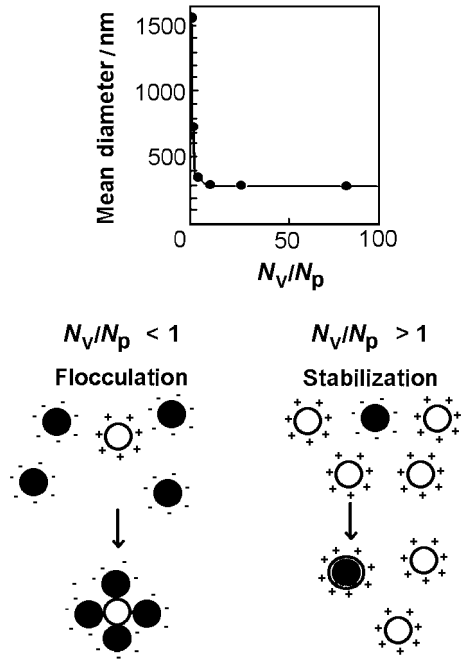

Fig. 3 The action of cationic bilayer vesicles composed of DODAC as flocculants or stabilizers of an oppositely charged dispersion of sulfate polystyrene microspheres. Particle size in the vesicle–particle mixtures is plotted as a function of the ratio between vesicles and particles number densities, N_v/N_p. Vesicles and particles are of similar sizes. Bilayer deposition causes flocculation when vesicles in the mixture are in the minority and particles the majority, whereas stabilization occurs when particles are in the minority and vesicles the majority.

stabilization of the dispersion. One should notice that the bilayer deposition event may or may not occur. Certainly, the magnitude of the electrostatic attraction between vesicles and microspheres plays a decisive role determining either bilayer coverage or mere vesicle adhesion to the microspheres. In Fig. 3 the mean particle diameter in the vesicle–particle mixture determined from dynamic light scattering is plotted against the number density ratio for vesicles and particles. Large DODAC vesicles (D_z = 256 nm) were mixed with sulfate polystyrene microspheres (D_z = 285 nm) 4 h before D_z determination. N_v is 8.05×10^{10} cm^{-3} as calculated from D_z for the vesicles and assuming 0.55 nm^2 per DODAC monomer. One week after mixing, flocculation was visible for samples in which N_v/N_p < 1 and absent when N_v/N_p > 1. Consistently, the new particle size measured for the stabilized system increased from 285 nm up to 295–296 nm as expected from deposition of a continuous bilayer of 5 nm thickness onto the microsphere surface. Later on we have shown that the interaction between bilayer vesicles and flocculated latex could lead not only to bilayer deposition but also to deflocculation of aggregated microspheres.[26]

Supported bilayers such as those on oppositely charged latex or on other polymeric surfaces (reference 26 and references therein) have also been obtained using hydrophilic silica particles as supports (reference 27 and references therein) though the silica surface shows different affinities towards DODAB, DHP and phospholipids (reference 27 and references therein). The affinity for neutral phospholipids in water or in 10 mM buffer is specially low in absence of Tris as buffer. As expected, at low ionic strength, the negatively charged DHP amphiphile adsorbs at the negatively charged silica surface with the lowest affinity whereas the positively charged DODAB exhibits the highest affinity.[28]

The hydrophobic/hydrophilic nature of bilayer and surface *and* the presence of electric charge on the solid support and/or on the bilayer may have a major role in determining the lipidic supramolecular assembly obtained at the solid surface. Whereas the negatively charged nature of sulfate polystyrene microspheres leads to deposition of cationic bilayers onto the surface, the positively charged nature of the amidine polystyrene leads to deposition of anionic DHP bilayers.[19] For neutral lipids such as phosphatidylcholine (PC) or dipalmitoylphosphatidylcholine (DPPC), the first deposition corresponds to a monolayer on the amidine latex with the phospholipid polar heads uppermost and the lipid hydrocarbon tails hydrophobically interacting with the latex surface.[20] Thereafter, upon increasing phospholipid concentration, additional bilayers may deposit due to the van der Waals attraction between the monolayer coverage and the free vesicle bilayers.[20,29]

4 Methods to quantify lipid adsorption onto particles from vesicles in dispersion

A valuable tool to quantify the amount of lipid adsorbed at maximal adsorption is the determination of adsorption isotherms. Fig. 4 is a scheme that illustrates the two important

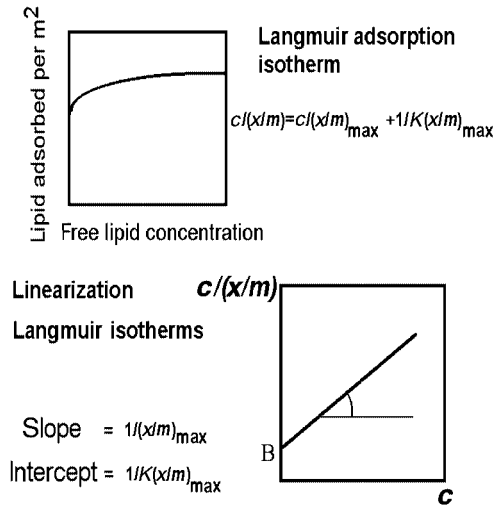

Fig. 4 Adsorption isotherms as an effective strategy to quantify deposition of lipids onto surfaces of large specific area such as those of small particles. If the adsorption isotherm is of the Langmuir type, calculation of the affinity constant (k) and the adsorption maximum $(x/m)_{max}$ is possible from linearization of the isotherm. This allows the comparison between different adsorbing lipids and adsorbate particles such as those in Table 1.

adsorption parameters that can be calculated from linearization of adsorption isotherms of the Langmuir type: affinity constant (k) and maximal adsorption $(x/m)_{max}$. The equation for linearization is given below taking $y = c/(x/m)_{max}$ and $x = c$ (see reference 2)

$$c/(x/m) = c/(x/m) + 1/[k(x/m)_{max}] \quad (1)$$

where x/m is the number of moles of amphiphile adsorbed per square meter of polystyrene, c is the free amphiphile concentration left in the supernatant after centrifugation of the vesicle/particle mixture and k is the affinity constant. The maximal adsorption $(x/m)_{max}$ can be obtained from the slope of the straight line given by the equation above whereas the affinity constant k can be calculated from the intercept.

Table 1 contains adsorption parameters for adsorption isotherms of lipids from vesicles onto a variety of model surfaces including those from bacterial cells. Determination of adsorption isotherms for lipids from liposomes onto latex, silica or bacteria followed by linearization of Langmuir isotherms and calculations of the affinity constants and adsorption maxima were shown to be valuable to ascertain the product of the liposome–surface interaction (Table 1). The occurrence of opposite charge for the liposome–surface pair generally drives

deposition of one bilayer onto the latex or the silica particle. Examples for this are the interactions between DODAB large or small vesicles and latex where the cationic DODAB deposits as bilayers onto the negatively charged sulfate polystyrene particles. Similarly, interactions between small or large DHP vesicles and oppositely charged amidine polystyrene particles leads to deposition of DHP bilayers onto the latex (Table 1). However, the *E. coli* cell, also negatively charged, does not interact with cationic DODAB or DODAC vesicles leading to bilayer deposition. There is mere adhesion of entire vesicles at the cell surface.[5] Given the roughness and complexity of the supramolecular assembly characteristic of the bacterial cell wall, which includes protruding sugars from glycolipids and lipoproteins, complex protein structures with multiple functions as recognition, adhesion, transport, *etc.*, it is not straightforward to achieve a simple explanation for the absence of cationic vesicle disruption at the cell surface. However, the usual hydration shells of biomolecules and bilayer vesicles could well provide an effective stabilization by short-ranged hydration repulsion forces that could prevent vesicle disruption upon its adhesion to the biological surface. Neutral phospholipids such as PC or DPPC from vesicles deposit as an odd number of monolayers onto the amidine latex surface (Table 1). This agrees with the interpretation of a first monolayer coverage on the solid surface with the phospholipid polar heads uppermost, deposition being driven by the hydrophobic attraction between lipid hydrocarbon chains and the hydrophobic surface.

A second possibility for detection of adsorption onto particulate surfaces is the determination of electrophoretic mobilities for particles in the vesicle–particle mixtures, a method that allows detection of change on particle charge that may take place when particles and/or vesicles are charged. For example, negatively charged bacteria[21] or mammalian cells[22] change the sign of their cell surface charge as a function of DODAB concentration, becoming less negatively charged by increasing concentration of this positively charged amphiphile. The interaction can be seen from the change of particle charge. Sometimes this change on particle charge can be related to another changing parameter such as cell viability or adsorbed amount, so that interesting conclusions are made possible. In the case of bacteria the occurrence of positively charged cells was related to cell death (total loss of bacterial cell viability) whereas the occurrence of negatively charged cells was associated with life.[30]

A third possible method to investigate the action of vesicles at particle interfaces is the determination of sizes in the particle–vesicle mixture as a function of the amphiphile concentration at a fixed particle number density for several values of particle number density. This works well for monodisperse particles such as the polystyrene microspheres that are not much larger than 200 nm mean diameter so that an increase in size of *ca.* 10 nm can be accurately measured, considering the experimental error of the light scattering technique. This increase in size can then be related to bilayer deposition onto the particle[19] and sometimes also to monolayer deposition.[20] Fig. 3 illustrates the bilayer deposition case where an increase in particle size of 10 nm was observed at N_v/N_p ratios larger than 1.

Recently a fourth method for quantitatively detecting bilayer deposition on small 50 nm silica particles was described that is based on the decrease in absorbance of a light absorbing dye that becomes 'sandwiched' between bilayer and particle upon bilayer deposition.[28] Reduction of merocyanine 540 absorbance at 565 nm was used as a marker for bilayer deposition onto silica particles. Upon interaction with the solid particle, absorbance at 565 nm decreases with time, corresponding to the percentage of dye that became sandwiched between the bilayer and the solid particle surface and thereby hidden from the incident light.[28] In this work, the percentage of bilayer deposition was calculated from the final absorbance reduction, with the final instant taken as 60 minutes after particle addition, as follows. The absorbance at 565 nm obtained for the dye after 1 h equilibration with vesicles alone will be called A_v. The absorbance at 565 nm obtained for the dye after 1 h equilibration with vesicles to which silica particles were also added at the instant zero will be called A_{v+p}. The absorbance reduction will be $\Delta A = A_v - A_{v+p}$. If all vesicle outer surface incorporating the dye were deposited as a bilayer coverage on the particles, the total absorbance reduction ΔA would be maximal and equal to A_v. If the vesicles were not interacting with the silica particles, no reduction would be observed so that ΔA would be equal to zero. If a fraction of the total vesicle number density were being used to cover the particles, the percentage of absorbance reduction would be $100\ \Delta A/A_v$. If all vesicles were covering particles, all *n* molecules of lipid in dispersion (known from the total amount of lipid in each sample) would originate an absorbance decrease equal to A_v. Therefore, an absorbance reduction equal to ΔA would require $n\ \Delta A/A_v$ lipid molecules and, from this, one can easily obtain the total number of lipid molecules that have

Table 1 Affinities (k) and maximal adsorption (x_{max}) for different liposome-forming amphiphiles onto a variety of organic, inorganic or biological surfaces. Models for organic synthetic surfaces are different types of latex (polystyrene) particles; for inorganic surfaces, conventional hydrophilic silica (Aerosil OX-50) and, for biological surfaces, *E. coli* cell surfaces. Liposomes were obtained from the following lipids: phosphatidylcholine (PC), dipalmitoylphosphatidylcholine (DPPC), dioctadecyldimethylammonium bromide (DODAB) or chloride (DODAC) or sodium dihexadecylphosphate (DHP). Latex particles are quoted as sulfate polystyrene (SP) or amidine polystyrene (AP) followed by the mean latex diameter in nm. Silica particles are Aerosil OX-50 (50 nm mean diameter) from Degussa

Liposome	Particle	Affinity, k/M^{-1}	$(x/m)_{max}/10^{-17}$ molecules per m^2	Lipid assembly at the surface	Reference
DODAB/SV	*E. coli* cell	23.1×10^4	200	Small vesicles adhered to the cell wall	5
DODAC/LV	*E. coli* cell	45.2×10^4	345	Large vesicles adhered to the cell wall	5
DODAB/SV	SP100	35.6×10^4	36	Bilayer	19
DODAB/LV	SP277	2.6×10^4	43	Bilayer	19
DODAC/LV	SP285	29.0×10^4	35	Bilayer	19
DHP/LV	AP850	75.0×10^4	53	Multibilayers	19
DHP/SV	AP97	51.0×10^4	55	Bilayer	19
PC/SV	AP104	50.0	15	Monolayer	20
		110.0	47	Monolayer + bilayer	
DPPC/SV	AP104	56.0	20	Monolayer	20
		—	64	Monolayer + bilayer	
DODAB/SV	Silica50	63.7×10^4	34–70	Bilayer + adhered vesicles	31
DPPC/SV		23.9×10^4	34	Bilayer	27
PC/SV	Silica50	2.0×10^4	32	Bilayer	27

adsorbed per m² of silica, in molecules per meter square. Fig. 5 illustrates the principle of the dye method that can be used to determine bilayer deposition onto large total surface areas of particulates.

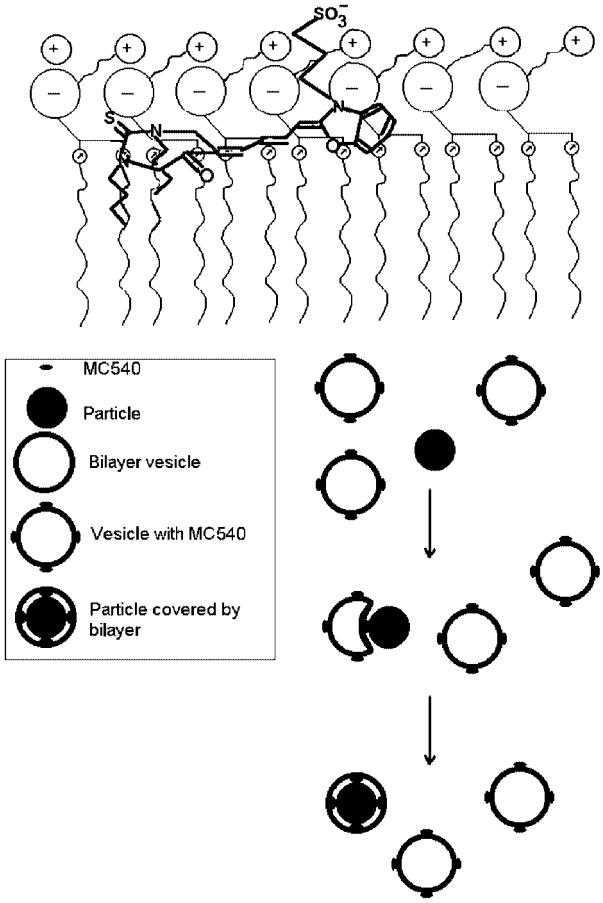

Fig. 5 A simple colorimetric method based on 'sandwiching' of merocyanine 540 in between the adsorbed bilayer and the adsorbent particle can be used to quantify bilayer deposition from the total decrease of dye absorbance upon mixing marked vesicles and particles. This was recently described in reference 28.

5 Reconstitution of biomolecular recognition using supported lipidic layers on particles

Fig. 6 illustrates functionalization of polystyrene amidine lattices in 3 steps. In the first, there is coverage of amidine polystyrene latex with the neutral phosphatidylcholine (PC) lipid from vesicles. At maximal adsorption *ca.* 40×10^{17} molecules per square meter polystyrene were obtained, indicating an area per molecule on latex of 0.25 nm² that is equivalent to 3 PC molecules occupying the mean molecular area of one PC molecule at the air–water interface, *i.e. ca.* 0.7 nm². Covering the microspheres with one monolayer plus one bilayer was the first of three steps to verify if these supramolecular systems could be used to amplify biomolecular recognition. At this point an adequate choice of a receptor for insertion in the outer PC layer is required. The monosialoganglioside GM1 was a very convenient option due to its avidity for the outer phospholipidic layers from micelle solutions. The chemical structure of this ganglioside shows its huge, hydrophilic and polysaccharidic polar head (the ganglioside is a glycolipid). The second step was the demonstration that GM1 could be incorporated onto the PC covered microspheres. The GM1 incorporation was detected thanks to the use of pyrenil covalently attached to the GM1 molecule so that the intensity of fluorescence emission left in the supernatant allowed quantification of the amount of GM1 attached to the PC-covered microspheres.[7] In the second step, maximal GM1 incorporation reached 50% of the total amount added when microspheres were covered with PC.[7] However, GM1 did not adsorb onto the latex by itself: there was an essential requirement of phospholipid molecules assembled on the microspheres in order to obtain GM1 insertion and toxin binding to the latex. The third step involves demonstration that the incorporated receptor is indeed functional in the artificial system. Toxin binding to GM1–phosphatidylcholine covered-polystyrene microspheres occurred in a molar proportion of 1:1 (toxin:GM1) and was strictly dependent on GM1 incorporation to the phospholipid covered-latex.[31] Therefore, phospholipid coverage on latex offers a proper environment to GM1–toxin recognition. Phospholipid assembly on the latex surface was also dependent on the physical state of the bilayer. A dipalmitoylphosphatidylcholine (DPPC) monolayer coverage on latex at room temperature can be obtained by incubating latex and DPPC vesicles in buffer solution for 1 h at 65 °C.[8] Furthermore, non-specific physical adsorption of cholera antitoxin on latex surfaces could also be controlled by covering latex with phospholipids.[8] Upon increasing dipalmitoylphosphatidylcholine (DPPC) concentration in latex dispersions, non-specific cholera antitoxin adsorption on latex decreases. This provided a way of modulating protein adsorption on the DPPC-covered latex.[8] Changing the hydrophobic–hydrophilic character of the antitoxin itself by covalent attachment of a *N*-acyl residue increases physical adsorption on bare latex and decreases it on phospholipid-covered latex. These results may be of importance in the development of immunoassays and biosensors for amplification of biomolecular recognition.[8,32]

6 Is there an ideal colloid available from coverage of polystyrene particles with an oppositely charged bilayer?

A corollary of the developments above is the preparation of the 'ideal colloid' (homodisperse polystyrene microspheres covered with an evenly charged bilayer membrane) useful for evaluation of current theories that predict colloid stability.[33]

Bilayer-forming amphiphiles assemble on latex depending on amphiphile type and concentration and on functional groups on latex (reference 26 and references therein). Neutral phospholipids have a specially high affinity for amidine polystyrene microspheres basically depositing as a phospholipid monolayer with the polar heads outermost[7,8,20] whereas the electrostatic attraction between cationic or anionic vesicles and oppositely charged microspheres may lead to deposition on latex of the bilayer membrane as a whole.[19,20,26,34]

Recently, the bilayer-covered assembly for dioctadecyldimethylammonium bromide (DODAB) bilayers on sulfate latex was used to evaluate the suitability of the DLVO theory for explaining the colloid stability of bilayer-covered polystyrene microspheres.[26] DLVO calculations show that experimental colloidal stability[34] is much lower than the theoretical colloid stability calculated from zeta-potentials and DLVO theory over a range of particle sizes and monovalent salt concentrations. The theory definitely does not explain the low experimental stabilities of the bilayer-covered latex. The key for a deeper understanding of these low colloidal stabilities may be found in the hydrophobic defects that result from the screening of the intralayer electrostatic repulsion upon salt addition.[33] In fact, the approach of adjacent polarheads at the outer monolayer upon addition of salt may well generate exposure of hydrocarbon chains and an extra attraction between different vesicles in dispersion.[33] Possibly, coverage with synthetic amphiphile layers such as those composed of DODAB or DHP, which form bilayer vesicles that are unstable at low concentrations of monovalent salt, are not adequate to produce coverages on solid

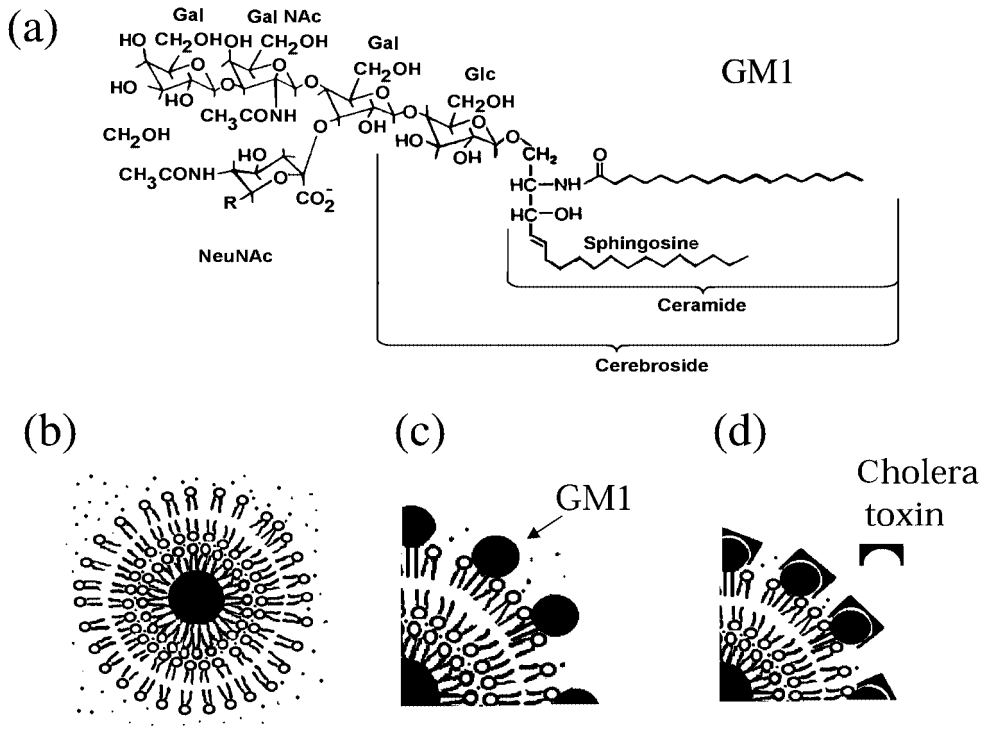

Fig. 6 (a) The monosialoganglioside GM1, a glycolipidic receptor of the cholera toxin and a very convenient molecule to represent an important class of receptors that can be inserted in a bilayer. (b) Starting a procedure to obtain microspheres functionalized with the GM1 receptor involves a sequence of essential steps. The first one is coverage of the microsphere with 3 phosphatidylcholine layers: one monolayer and one bilayer (as quantified from the adsorption isotherm in reference 7). (c) The second step is spontaneous assembly of the receptor GM1 in the outer phospholipid layer. The amount inserted was quantified from the fluorescent-labelled GM1 left in the supernatant and GM1 insertion was clearly dependent on previous coverage of the microsphere with the phospholipid.[7] (d) The third step is the determination of binding of the cholera toxin to the GM1 receptor contained in the functionalized microspheres. The protein binding was specific and obtained under conditions of excess of GM1 on the surface of the functionalized microspheres. Every protein molecule added becomes bound under these conditions thus yielding a linear dependence between binding and amount of added protein.[8]

surfaces, where hydrophobic defects would be absent. We are presently trying other lipids in our lab that might be able to produce coverages with effective hydration layers as stabilizers so that we can finally test the DLVO theory with ideal model colloids.

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