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Vesicle-forming synthetic amphiphiles

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1. Introduction

1.1. History

Cellular membranes are extremely complex, integrate supramolecular structures which effectively control the passage of materials into and out of the cell. Membranes also provide an efficient organization of life processes by

compartmentalization. Lipids and proteins (both 'trans-membrane' and 'peripheral') are the main constituents. In addition carbohydrate structures are exposed on the exoplasmic side of the PM as crucial parts of glycolipids and glycoproteins, which are involved in important cellular activities. Eversince the development of influential models for the biomembranes, including the Danielli-Davson model [1] and the fluid mosaic model [2], it has been generally agreed that the lipid bilayer structure must be viewed as the structural basis of the biomembrane [3]. This matrix is formed from a mix of various classes of lipids, including (glycero)phospholipids, glycosphingolipids,

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Fig. 1. Structural formula of some vesicle-forming synthetic amphiphiles.

glyco-glycerolipids and sterols. The specific role of the different components for various cellular functions has only been partly identified and many important issues concerned with lipid specificity are not understood. In view of this situation, it is obvious that there has been and still is great interest in studies aimed at developing model membranes of much less complexity which could assist in acquiring further insights into aspects of essential bilayer properties and functions. Bangham's pioneering work [4] showed for the first time that closed bilayer vesicles are formed from aqueous dispersions of phospholipids if mechanical energy is supplied to the system. These *liposomes* have gained an enormous popularity as simple model membranes and their physics and chemistry has been studied in great detail [5]. Interestingly, in later studies indications were found that the typical phospholipid structure is by no means a prerequisite for vesicle formation. The definite study came from Kunitake et al. [6] who showed convincingly that the clear aqueous dispersion of di-*n*-dodecyltrimethylammonium bromide (DDAB; Fig. 1), formed by sonication, contained uni- and multilamellar vesicles with a bilayer thickness of 30–50 Å. This *totally synthetic bilayer* membrane marks the birth of 'membrane mimetic chemistry' [7]. In an outburst of subsequent research activity it became clear that a large variety of synthetic amphiphiles in aqueous solution can form open, flat lamellar bilayers and/or closed vesicles. The properties of the vesicles usually resemble those of phospholipid vesicles (liposomes). Structural factors in the amphiphile responsible for self-organization in bilayers have been identified in some detail and even spontaneous vesicle formation in water was observed under special conditions [8]. Structural variations included length of the alkyl chain(s) (usually C₁₀–C₂₀), chain branching, headgroup structure (cationic, anionic, zwitterionic, (di)polar; more than one headgroup, multivalent headgroup(s)), and introduction of spacers and rigid fragments. The effects of these

structural changes on the properties of the vesicles were studied and allowed 'tuning' of the vesicular structure for specific applications [9]. Bola-amphiphiles represent another example of an important class of vesicle forming synthetic amphiphiles. These 'double-headed' single-chain amphiphiles may mimic the chemistry of membrane lipids of certain thermophilic and acidophilic archaeobacteria. Their unusual self-organization has been studied in detail by Fuhrhop et al. [10]. Polymerized bolaform surfactants exhibit a high membrane-disrupting capacity [11]. Relatively stable, multilamellar nonionic vesicles (niosomes) can be obtained from, e.g., C₁₆-polyglycerolether (PGE; Fig. 1) and related structures [12].

The size of synthetic amphiphile vesicles can vary substantially (30–800 nm) and a single vesicle may easily contain 10⁵–10⁶ monomers. Recently, Menger et al. [13,14] described procedures for the preparation of giant unilamellar vesicles with diameters in the range of μm and which are well observable under the light microscope. The study of their highly interesting cell-like activities (fusion, fission, endocytosis, budding, aggregation, birthing, and foraging) has been termed 'cytomimetic chemistry' [14].

The vesicle membrane can consist of a single ('unilamellar vesicle') or multilayered ('multilamellar vesicle') double-layer of amphiphilic molecules. Accordingly, the sizes of the aqueous inner compartments can be varied greatly and typically have diameters of 20–100 nm. In attempts to increase the vesicular stability and to control size, shape and bilayer permeability, polymerized synthetic surfactant vesicles have been prepared [17]. The polymerizable functional moiety in the surfactant molecule has been incorporated in the headgroup or in the alkyl chain at different positions relative to the headgroup. Indeed, polymerization was found to lead to greatly improved stabilities and otherwise modified the properties of the vesicles substantially. Since their relevance for mimicking properties of biological membranes is not immediately apparent these polymerized vesicles as well as polymeric vesicles [15] will not be further discussed.

Thermal stabilities of the vesicular dispersion vary widely and depend on the structure of the amphiphile. Physical properties of the vesicle bilayers have been investigated in some detail with particular emphasis on the molecular organization in the core of the bilayer (Section 2.1). The physics and chemistry of vesicle formation from the solid surfactants is a challenging and interesting issue [16] and recently some imaginative comments were made on the how and why of vesicle formation [17].

Synthetic amphiphile bilayers can serve as matrices for membrane protein reconstitution [18], they can undergo fusion and they are osmotically sensitive. In fact, the properties appear remarkably relevant for obtaining a better understanding of fundamental aspects of the much more complex natural membranes. Furthermore, a wide range of industrial applications has been envisaged [19].

Moreover, the significance of applying synthetic amphiphiles as (drug) carrier systems is becoming increasingly apparent. The validity of this potentiality is supported by observations that the most efficient transfection systems are based on the use of synthetic amphiphiles as carriers for nucleic acid [20–22] (see Section 3.3).

This review will not provide an exhaustive discussion of the vast literature on synthetic bilayer-forming amphiphiles. A comprehensive summary of the literature up to the early eighties can be found in Ref. [7]. We will focus on those aspects which are most relevant in the context of modelling structure and properties of biological membranes. Issues to be discussed include fusion, molecular recognition and binding and, finally, catalysis. Inevitably, the discussion will be arbitrarily biased to the work in the authors' laboratories. For further reading, the reader is referred to several excellent reviews [23–25] addressing some topics which will not be discussed here.

1.2. Molecular organization of synthetic amphiphiles in water

Surfactants usually form three-dimensional molecular aggregates in water. The main driving force is hydrophobic interaction; the alkyl chains are buried in the core of the aggregate to avoid direct contact with water [26]. This would suggest that aggregation is limited to water as the solvent. However, a distinct molecular assembly also takes place in other liquids of high cohesive energy density like hydrazine, formamide and ethylene glycol and in liquid electrolytes like ethylammonium nitrate [27]. The size and morphology of the amphiphile assembly is governed by a complex compromise of intermolecular noncovalent interactions leading to a minimum in Gibbs energy. The process has been analyzed most rigorously in terms of equilibrium thermodynamics (Section II-B). Recently, the development of high-speed computers has allowed meaningful computer simulations of micelles [28] and bilayer vesicles [29,30]. These simulations in conjunction with spectroscopic studies [31], have clearly shown the highly dynamic nature of the core and, even more significantly, of the surface structure of the aggregate. Many attempts have been made to rationalize the preference for a certain type of morphology of a particular surfactant under well-defined conditions: spherical or wormlike micelles, bilayers (both planes and closed) and inverted hexagonal and inverted cubic structures. Even more complex structures are possible, particularly in surfactants carrying chiral moieties in the headgroup region of the molecule [32]. A most useful approach in correlating surfactant structure with aggregate morphology has been couched in terms of geometric shape constraints. Thus, a dimensionless critical packing parameter P has been defined [33] with a magnitude which depends on the volume v of the fluid and incompressible hydrocarbon chain(s), the cross-sectional headgroup area (a_0) per surfactant molecule exposed to

the aqueous phase and the 'critical chain length' (l_c ; $\sim$ maximum effective chain length). For a critical

$$P = v/a_0 \cdot l_c$$

discussion of this parameter, the reader is referred to the original literature [34,35]. In a good approximation, v and l_c for a saturated hydrocarbon chain of n carbon atoms is given by [36]:

$$v \approx (27.4 + 26.9n) \text{ \AA}^3$$

$$l_c \approx l_{\max} \approx (1.5 + 1.265n) \text{ \AA}$$

Specific values for P are associated with spherical micelles ($P < 1/3$), wormlike micelles ($1/3 < P < 1/2$), vesicles ($1/2 < P < 1$), planar bilayers ($P = 1$) and inverted micelles ($P > 1$). This approach has remarkable predictive power under conditions of thermodynamic equilibrium. An example is provided by the recent finding that *n*-octyl 1-thio- α -D-talo-pyranoside forms vesicles in water whereas other glycopyranosides with only one *n*-octyl chain form micelles. This difference in morphology can be explained in terms of the smaller effective cross-sectional headgroup area of the D-talose surfactant because of the intramolecular hydrogen-bond formation between OH-2 and OH-4 which reduces the size of the hydration shell (van Doren, H.A., Galema, S.A., Engberts, J.B.F.N., Langmuir (1995) 11, 687,688). However, we stress that ab initio estimation of v , a_0 , and l_c is not a trivial problem. Few systematic tests [37] of the theory have been published and it should be noted that the approach has been questioned [38]. Apparent exceptions to the rules have been reported [39,40]. Attempts have been made to rationalize the deviant behavior in terms of interdigitation of the alkyl chains in the bilayer [40]. Of course, P values a priori cannot take into account different types of interdigitation and tilting of the alkyl chains in the aggregate. The morphology of aggregates of surfactants of more complex structure (like gemini surfactants [41]) is still difficult to predict. If we restrict our discussion to vesicular bilayers ($1/2 < P < 1$), it is obvious that most of the vesicle-forming amphiphiles contain two alkyl chains. For these surfactants, the volume of the chains is just too big to allow packing in micellar structures while maintaining an optimal value for a_0 . The exact curvature of the bilayer and size of the vesicles is related to the magnitude of P . Gradual addition of the single-chained *n*-dodecyltrimethylammonium bromide to a lamellar phase of di-*n*-dodecylmethylammonium bromide (DDAB; Fig. 1) has interesting consequences. First the lamellar phase disperses into polydispersed vesicles. These vesicles, in turn, are transformed into rod-like micelles until finally spherical micelles are obtained [42]. Interestingly, however, several specific types of single-chain amphiphiles may exclusively form vesicles, provided that the alkyl chain is branched [43,44]. The magnitude of P is further affected by factors like unsaturation in the chain(s), pH, and temperature

which influence the motion of the apolar chains and the hydration of the headgroup. In the context of this discussion, we note that for short-chain amphiphiles more than one type of aggregate morphology may exist under particular conditions (e.g., micelles and vesicles). The presence of electrolytes can also have a dramatic effect on the aggregation process [45]. A related approach for understanding the structure-morphology relationship is the spontaneous-curvature model [46,47]. In this theory two counteracting forces are taken into account. One is the tendency of certain lipid monolayers to curl and the other is the packing of the alkyl chains which opposes bending of the biomembrane. The hypothesis is that biomembranes adjust their intrinsic curvatures to fall into an optimum range. For example, bilayers may be destabilized by protein-mediated introduction of hydrophobic molecules.

2. Aspects of membrane biomimetic chemistry

2.1. Properties of vesicular bilayers

Generally, vesicular dispersions are prepared by methods also employed for liposome preparation. Procedures include sonication (in a bath or with a tip), reverse phase evaporation, ethanol injection etc. The size and presumably also the shape, of the vesicles are determined by the preparation method. This also applies for thermal stability (*vide infra*). Vesicles can also be prepared by stirring surfactant crystals in water (or buffer) at an appropriate temperature (see also Section 2.2).

Bilayer and colloidal stability of aqueous dispersions of synthetic amphiphile vesicles depend on the inherent (thermodynamic) stability of the vesicle and on different types of repulsive intervesicular forces, including hydration forces and repulsive interactions due to thermal fluctuation forces associated with the fluid-like nature of the bilayer surface. The latter type of repulsive forces may originate from protrusion, undulation and peristaltic fluctuations [48]. Thermal fluctuations of biological (model) membranes have been analyzed in some detail [49]. It should be noted that vesicular dispersions are often metastable and that their thermal stability may depend critically on the mode of preparation. Often there is a tendency for flocculation and crystallization, depending on the nature of the surfactant monomer in the bilayer. The nucleation for crystallization of sodium di-*n*-dodecyl phosphate vesicles appears to take place at the water-air interface. Reducing the specific water-air surface area leads to increased colloidal stability.

Vesicle aggregation and fusion (Section 3) are also important phenomena in determining vesicle stability, but the exact nature of intervesicle adhesive forces appears to be not well understood. Prolonged storage of vesicle suspensions without the occurrence of aggregation and precipitation can further benefit from an appropriate choice of the counterion and from increasing the temperature above

the main phase transition temperature. The structural properties of vesicles formed from synthetic amphiphiles are characterized by a variety of well-established methodologies, also used for the study of liposomes. Electron microscopic techniques are of particular importance. Time-resolved cryotransmission electron microscopy shows great promise for investigating changes in bilayer microstructure during dynamic processes like phase transitions and chemical reactions [50]. Other novel methods include scanning tunneling microscopy and atomic force microscopy [51]. Bilayer dimensions and structures are obtained from X-ray data and from ^1H , ^2H , and ^{13}C -NMR data. Vesicular bilayers formed from synthetic amphiphiles undergo thermotropic phase transitions and also in this regard, their behavior resembles that of liposomes. For example, di-*n*-octadecyldimethylammonium chloride (DODAC) vesicles exhibit a pretransition and a main phase transition characterized by the corresponding phase transition temperatures [52]. Similar phase transitions have been studied for many vesicular bilayers and the effect of structural changes in the synthetic amphiphile on these transitions has been investigated. A comparison of the main phase transition temperatures has been made for a series of anionic, cationic, nonionic, and zwitter-ionic headgroup surfactants with a fixed alkyl chain length. It was concluded that the anionic headgroup produced the most rigid bilayer surfaces [53].

Main phase-transition temperatures have been examined for a large series of identical and mixed-chain di-*n*-alkyl phosphate vesicles [54]. Generally the T_c values decrease with decreasing chain lengths and increasing asymmetry of the alkyl chains. The T_c values reflect the efficiency of alkyl chain packing in the bilayer and the structural effects appear to resemble those in phospholipid vesicles. The effect of counterion binding on the T_c values for the dialkyl phosphate vesicles has been studied and was found to be significant only for the amphiphiles with the longer alkyl chains ($\geq C_{14}$) [55]. Fluorescence depolarization (using a rigid fluorescent probe) and, perhaps providing the most detailed information, differential scanning microcalorimetric techniques (Section 2.2) have been employed to characterize the phase of the bilayer at a particular temperature. These insights in aggregate polymorphism are of great importance for understanding vesicle fusion (Section 3) [56]. Bilayers, composed of amphiphiles containing an azobenzene moiety in the alkyl chain, can undergo photo-isomerization. Since the phase behavior of the bilayer is a crucial element in these processes, these lamellar aggregates offer possibilities for the construction of artificial photosynthetic systems [57]. Lateral diffusion and flip-flow movement of amphiphiles in the bilayer have also been examined [58,59]. The headgroup conformation of DDP vesicles has been assessed using ^{31}P -NMR and an order-parameter approach [60]. The two nonesterified oxygen atoms of the phosphate moiety are favorably located at the bilayer surface for interaction with cations and for hydrogen-bonding interactions with water. The DDP

molecules not only rotate around their long axis, but the long axis itself performs a wobble within a cone which makes an angle of ca. 55° with the bilayer normal.

In this brief survey we finally mention the capacity of synthetic amphiphile vesicles to display osmotic activity [61]. For example it was shown that large vesicles formed from sodium di-*n*-hexadecylphosphate (DHP; Fig. 1) and dioctadecyldimethylammonium chloride in the gel state display a highly selective permeability. Water, ethylene glycol, and ammonium acetate (in the form of NH₃ and acetic acid since all tested ionic solutes were impermeant) permeate across the bilayer, but numerous other solutes do not, including urea. In the liquid-crystalline state, large DHP vesicles responded as ideal osmometers towards gradients of sucrose, NaCl, and NaOH.

2.2. Thermodynamics

Differential scanning calorimetry (DSC) is a particularly attractive method for the measurement of the (main) phase transition temperature (T_c) of vesicular bilayers. In the DSC experiment, the differential heat capacity of an aqueous vesicle dispersion relative to that of pure water is monitored accurately. The experiment not only provides T_c , but also the calorimetric enthalpy of the melting transition and the patch number. The last quantity entails the cluster of monomers in the bilayer undergoing the melting process cooperatively [62]. Its value is obtained from a comparison of the calorimetric with the van 't Hoff enthalpies [62]. For example, for a highly dilute ($1 \cdot 10^{-4}$ M) dispersion of dioctadecyldimethylammonium bromide (DOAB) vesicles, T_c is 45°C and the patch number is about 130. For anionic di-*n*-dodecylphosphate (DDP; Fig. 1) vesicles, T_c is 35°C and the patch number amounts to ca. 170 [63]. For the DDP vesicles, definite evidence was obtained that preparation of the vesicles by the popular ethanol-injection method leads to additional phase transitions, apparently induced by the presence of ethanol. A single T_c is obtained when the vesicles are prepared by the hot-water method.

The amphiphile concentration is an important issue in these experiments. In less dilute solutions, often an additional transition is manifested [63] and the patch number tends to decrease with increasing amphiphile concentrations. It is anticipated that insights into the phenomena will greatly improve the understanding of structural and physicochemical changes relevant to intermembrane interaction.

In mixtures of vesicles formed from two di-*n*-alkylphosphates with different alkyl chain lengths, evidence has been obtained for two patches of different size undergoing the phase transition cooperatively. Consistent with results obtained by other techniques (e.g. fluorescence depolarization, Section 2.1) T_c increases with increasing alkyl chain length in di-*n*-alkylphosphate vesicles (unpublished). Other DSC studies include the measurement of

interaction enthalpies for binding of (divalent) anions to DOAB vesicles [64]. Preliminary studies of vesicle fusion (section III) have been performed using a titration microcalorimeter (unpublished).

2.3. Vesicle fusion

It has become apparent that vesicles, prepared from a variety of synthetic amphiphiles, may serve as valuable models for studying fundamental aspects of membrane fusion. The alkyl chains are easily amenable to chemical modification with regard to length, branching and saturation. Moreover, since the structure that connects the hydrophobic chains and hydrophilic headgroup can also be modified, thereby determining headgroup mobility and orientation, an overwhelming number of amphiphiles are potentially available that would allow a systematic study of parameters that are relevant to the mechanism of membrane fusion. The documented polymorphic properties of synthetic amphiphiles are of particular interest in this context, as will be discussed below. Membrane fusion is vital in many biological processes, occurring during intracellular flow of transport vesicles in endocytic and exocytic pathways, upon infectious entry of enveloped viruses into host cells, but also between sperm and oocyte during fertilization [65–67]. The overall fusion event can be seen as a sequence of a second-order process involving aggregation or interlamellar attachment(s) between adjacent membranes, which is followed by the first-order fusion reaction itself [68]. Important features are the molecular factors that bring about the close approach of membranes and those related to the departure of the membrane from its bilayer structure, a transient destabilization essential for apposed membranes to merge. Although well-known as a molecular event for several decades, the molecular basis of the mechanism of fusion remains ill-defined, particularly with regard to the relevance of lipid polymorphism in the merging process [69].

Vesicles composed of anionic or cationic amphiphiles, either pure or mixed with phospholipids, are able to fuse. Fusion is triggered by adding anions such as citrate, EDTA, dipicolinic acid dianion (DPA²⁻) and sulphate to cationic vesicles, or divalent cations to anionic vesicles, [70–74]. Although monoanions can readily cause the aggregation of vesicles composed of either anionic or cationic amphiphiles, they fail to induce fusion [75]. However, vesicles composed of the cationic amphiphile DDAB have been shown to engage in fusion induced by monoanions that also contain a hydrophobic group, such as the anions of benzoic acid and *p*-toluenesulfonic acid [70]. These observations bear relevance in the context of the long-standing concept of the role of hydrophobic interactions as a key-feature of the fusion mechanism [76–78]. Such interactions result from local membrane dehydration, arising from either water displacement upon binding of the fusion-inducing ion and specific complexation with the

binding site (dehydrated 'trans'-complex formation between ion and lipid/amphiphile [77,78]) or in case of biological membrane fusion, the interaction of hydrophobic protein segments with the target membrane [65,66]. Experimental support of the latter event has mainly been derived thus far from work carried out with viruses [65,66,79,80].

To investigate the mechanism of membrane fusion, it is important to register the event kinetically, in a direct manner, so that structural and/or physical changes in or at the membrane can be correlated to the cause or consequence of the fusion process. This has been done by applying fusion assays, in particular those based upon lipid mixing [81–83]. Application of contents mixing, in conjunction with membrane mixing reflecting the most reliable approach to appropriately assess fusion, is commonly precluded [70,84,85], partly due to the inherent fusogenic nature of multivalent cations and anions such as terbium, citrate or aminonaphthalene trisulfonate, representing ions which are employed in such assays. Furthermore, pure membranes consisting solely of synthetic amphiphiles are often found to be less stable than phospholipid bilayers, resulting in the release of vesicle contents. This property, however, can be exploited in the development of amphiphiles as drug carriers (see below). Therefore, in addition to lipid mixing, electron microscopy is not just an alternative, but also a highly recommendable approach for determining the occurrence of fusion per se in such systems [86,87].

2.4. Mechanism of fusion.

In a series of studies, Rupert et al [56,70] have examined in substantial detail the mechanism of fusion of cationic (DDAB) and anionic (DDP) vesicles of synthetic amphiphiles. Approaches based on NMR and fluorescence techniques, using ANS and DPH to monitor surface and intrabilayer changes upon interaction of the bilayers with fusogenic anions and cations, respectively, revealed that fusion is conferred to the vesicles in the aggregated state, implying fusion itself to represent the rate-limiting step, and that the bilayers should be in the liquid-crystalline phase. The physical state of such a bilayer, like that formed by DDP, can be strongly affected by the pH, which, consequently affects the fusion event. Thus, the bilayer fluidity, and hence the fusion activity are minimal at the effective pK_a of the DDP headgroup [71]. As for phospholipid systems [77,78], hydration repulsion is a governing factor in the mechanism of fusion of vesicles prepared from synthetic amphiphiles. Furthermore, since the ion threshold concentrations required for aggregation and fusion of DDAB [70] and DDP [86] vesicles are quite distinct, a fairly detailed molecular scenario can be obtained as to the physical and structural factors pertinent to aggregation and those that are relevant to fusion. Using NMR spectroscopy, it has been shown that Ca²⁺ binding

to DDP results in a dehydration and a concomitant reduction of the mobility of the headgroup, *well below* the threshold concentration for aggregation, i.e., on separate vesicles [86]. Similarly, the dianion of dipicolinic acid (DPA²⁻) can bind to separate DDAB vesicles, resulting in decreased electrostatic repulsion and dehydration of the bilayer surface [70]. Interestingly, the physical and structural alterations induced in DDAB bilayers upon binding of DPA²⁻ are entirely restricted to changes at the bilayer surface, i.e., fusion does not require the induction of an isothermal phase transition. The events as described are taking place within the lateral plane of the same bilayer rather than between apposed bilayers. Initial interaction between amphiphile and fusogenic agent thus results in formation of a so-called 'cis' (intrabilayer) complex, that causes the vesicles to aggregate due to electrostatic screening of the surface charge, analogous to divalent cation-induced aggregation of phospholipid vesicles. Subsequent fusion occurs for both DDAB and DDP vesicles well above the threshold ion concentration for aggregation, implying that an additional process has to take place to trigger the actual fusion step. It is thought that this step involves the formation of an *interbilayer*, 'trans' complex in which the fusogenic agent is complexed between two headgroups in adjacent bilayers of a vesicle aggregate. Possibly, such a complex will induce a coordinated perturbation in apposed bilayers at sites of intimate contact, in turn triggering the merging process. Whether such a coordinated perturbation leads to the formation of a defined intermediate lipidic structure [69] will be discussed below. However, that such a perturbation can be restricted to a *local* perturbation has been suggested by experiments and calculations which showed that for the DDP/Ca²⁺ system about half of the phosphate headgroups are involved in the formation of a 'cis' complex before aggregation can occur [86]. Most interestingly, there is a relatively minor increase of only 6% in the fraction of complexed headgroups necessary in order to render the vesicles susceptible to fusion. This increase likely reflects the fraction that is involved in creating the local contact sites for fusion, which is accomplished by a local enhancement of Ca²⁺ binding and the formation of a (dehydrated) 'trans' complex that triggers the actual fusion process. It is possible that titration microcalorimetry (Section 2.3) may provide an important tool in elucidating further molecular and physical details of this process.

It appears that hydration represents a crucial interfering force in membrane fusion. Like systems consisting of phospholipids, amphiphilic systems as described here experience a repulsive force depending on the state of hydration. Modulation of the hydration shells, for example by treating the system with dehydrating agents such as polyethylene glycol (PEG), strongly facilitates membrane fusion of synthetic amphiphilic vesicles [88]. However, the effect of PEG is strongly dependent on the molecular weight of the polymer and its concentration (for a detailed

discussion, see Ref. 88). Furthermore, strongly hydrated phospholipids such as PC, inhibit the fusogenic properties of amphiphile-containing bilayers [72,74]. Another aspect is the binding affinity between amphiphile and fusion-inducing agent and whether such an interaction suffices to form a sufficiently dehydrated 'trans'-complex between an amphiphile and a particular fusion-inducing ion. For example, the dianion of dipicolinic acid and sulfate induce fusion of DDAB vesicles, but not of DOTMA-containing vesicles (Fig. 1) [74]. Mg^{2+} displays a higher binding affinity for DDP vesicles than for PS vesicles. Yet, as for PS vesicles, Mg^{2+} does not trigger the fusion of DDP vesicles. Since Mg^{2+} is more hydrated than Ca^{2+} [89], the results emphasize the need for a dehydrated complex to form before fusion can be triggered. In this context it is further of interest to note the distinct differences between the state of headgroup hydration and the differences in ion concentration needed to bring about fusion by displacement of the hydration layer upon binding to the amphiphile. A phosphate headgroup is more strongly hydrated than a dimethylammonium headgroup as reflected by both the number of water molecules (4–5 and 2, respectively) in the first hydration shell, and the difference in hydration energy per water molecule [90–92]. In conjunction with findings that long-chain analogs of DDAB experience only a minor hydration force relative to the electrostatic repulsion [93,94], while vesicles prepared from amphiphiles with a phosphate headgroup are frequently found to be more stable in high salt than those with a ammonium headgroup [75], existing evidence would thus favor the notion that DDAB head groups are more readily dehydrated than DDP head groups. Observations that DOTMA-containing vesicles fuse spontaneously with PS vesicles [74] can be explained along these lines in that electrostatic interactions between the headgroups suffices to dehydrate the ammonium headgroup to such an extent that the molecules readily adopt a fusion triggering conformation (see below). Apart from a mechanistic point of view, this observation is also relevant in the context of 'asymmetric' fusion, i.e., conditions under which nonalike vesicles fuse with each other. Fonteijn et al [72] have

shown that a variety of synthetic di-*n*-alkylphosphate vesicles can readily engage in asymmetric fusion with phospholipid vesicles, provided that the target membrane for the amphiphilic vesicles is in a fluid state (Fig. 2). The latter would indicate the importance of alkyl chain mobility in the fusion process. No particular requirements have to be met with regard to polymorphic properties of the target membrane lipids. Apparently, the nonbilayer structure that amphiphiles such as DDP readily adopt [16,86], suffices to destabilize the 'asymmetric' DDP-phospholipid interaction site to an extent that triggers the fusion of the membranes. Previously, it has been hypothesized [95] for vesicles containing HII phase forming phospholipids that when the lipids of two dissimilar liposomes (A and B) are HII competent, the asymmetric bilayer destabilization (AB) is more important than either of the two symmetric destabilization processes (AA and BB).

An inherent part in elucidating the mechanism of membrane fusion involves the identification of intermediate fusogenic structures, i.e., the sequence of structural transformations in a local region within the membrane arising from interaction between fusogen and the membrane. The analysis of such structures is hampered by the high Gibbs energy and thus the short-lived nature of these intermediate structures and the inability thus far to fix such transition events [96]. Intermediates that have been proposed to play a key role as fusion intermediate include inverted micellar structures, i.e., spherical micelles contained within a semi-toroidal outer monolayer connecting the interfaces of two original bilayers [69]. The involvement of so-called stalks has also been proposed [97,98]. These highly bent structures represent catenoidal connections between the interfaces of apposed bilayers, the formation of which is inhibited when molecules with a shape of inverted cones, such as lysophospholipids, are present in contacting leaflets [98]. Several synthetic amphiphiles, in particular di-*n*-alkylphosphates, have shown to display a very dynamic polymorphic behavior [86,88]. The polymorphic dynamics may well be related to the low state of hydration of amphiphiles (see above) when compared to that of phospholipids and given the effects hydration exerts on the

Fig. 2. Electron micrographs of asymmetric PS-DDP and DOPC-DDP fusion products. A and B show the asymmetric mixture of PS and DDP vesicles (ratio 1:1), before (A) and after (B) addition of Ca^{2+} . (C) A 1:1 mixture of DOPC and DDP vesicles before addition of Ca^{2+} . Electron micrograph D is obtained after addition of Ca^{2+} . The bars represent 300 nm.

Fig. 3. Negatively stained electron micrograph of a NaDDP crystal suspension after stirring in aqueous 0.3 M NaCl solution: tubular structures. Bar represents 1000 nm.

metastability and structures of lipids [99–101]. It has been reported that in the hexagonal phase, the phosphate group of PE has a weaker shell of hydrogen-bonded water than in the lamellar phase [99], while direct evidence for a partial dehydration of PE bilayers on approaching the hexagonal phase, has recently been presented [101]. For Ca^{2+} -induced fusion of DDP vesicles, it has been suggested that these vesicles fuse via the formation of an unstable inverted micellar intermediate (IMI). According to this mechanism, as originally proposed by Siegel [102], these transient structures within an interbilayer attachment site may either revert or transform into an interlamellar attachment (ILA), a fusogenic structure arising when the IMI remains isolated. When the number of IMI's increases, adjacent IMI's may aggregate and merge into an HII phase via coalescence of IMI's in a pearl-string fashion or via

formation of line defects (LDs; Ref. 69), originating from pairs of IMI's. In fact, the large vesicles that are formed upon addition of Ca^{2+} to DDP vesicles are unstable, and transform into long tubular structures in which the DDP molecules have been claimed to be arranged in an hexagonal HII phase as revealed by cryo electron microscopy, electron diffraction and NMR analysis [86]. More recently the hexagonal phase identity of the observed tubes has been questioned as X-ray analysis indicated that the tubular structures are organized in lamellar crystals [103] (Fig. 3). However, as long as the distinct transitions cannot be arrested and the particular complexes analyzed under controlled conditions, it cannot yet be excluded that the crystals arise from initial processes that involve defined non-lamellar structural transitions (IMI, stalks, etc.). Therefore, the exact identity of the fusogenic intermediates remains a

Fig. 4. Myelin formation at room temperature of tetramethylammonium di-*n*-dodecylphosphate in aqueous solution. Bars represent 100 μm .

matter of speculation. In fact based on kinetic criteria and cryo electron microscopic studies, lamellar/inverted phase transitions are more likely to proceed via formation of a stalk like structures than via IMIs, implying that the IMI concept needs some revision [97–101,104]. Regarding the present uncertainty of the structural features of the fusogenic complex, additional work [16] has revealed that the polymorphic behavior of DDP is highly complex and can also result under distinct temperature-dependent conditions in the formation of myelin tubes (Fig. 4). Addition of Ca^{2+} results in the formation of similar crystalline complexes as observed for the unstable fusion products, suggesting that the crystals may represent a structure present at equilibrium conditions. Nevertheless, it appears that synthetic amphiphiles can assist in solving the structural nature of fusion intermediates. An important feature involves the propensity of at least some amphiphilic vesicles that the transition under fusogenic conditions from separate vesicles to aggregated structures and subsequent formation of the fusion product, is better controllable than that of phospholipid vesicles, for which the sequence of events is virtually instantaneous.

2.5. Synthetic amphiphiles as carriers. Interaction with cell surfaces

Recently synthetic amphiphiles have attracted much attention as a convenient polynucleotide transfection reagent. This application is particularly relevant in the context of genetherapy, which is one of the fastest growing areas in biomedical sciences. For efficient transfer of foreign genes into target cells, both viral and nonviral vectors have been developed in the quest for the perfect gene delivery system. However, cationic carriers, composed of synthetic amphiphiles seem particularly attractive for transfer purposes, offering considerably advantages over viral vectors or liposomal carriers because (i) the efficiency of delivery is usually very high, (ii) they are non-immunogenic, (iii) easy to use and (iv) give rise to a relatively low toxicity. It has thus become apparent that synthetic amphiphiles provide, compared to conventional techniques, a superior and highly efficient technology for cellular transfection with recombinant DNA vectors. Particularly notable is the original application using DOTMA/DOPE mixtures, commercially known as lipofectin. Transfection of cultured cells has been accomplished with different classes of polynucleotides, including DNA, mRNA and double stranded RNA [105,106]. To further diminish the toxicity, occasionally noted when applying DOTMA, to increase the potency of transfection or to transfect cells that were not susceptible to DOTMA, other amphiphiles have been synthesized and tested. Invariably, the successful amphiphiles in terms of delivery efficiency are of a cationic nature, containing an ammonium head group [21,107–112]. This point suggests the relevance of an electrostatic interaction between am-

phiphile (positively charged nitrogen) and nucleic acid (negatively charged phosphate).

From the point of view of an efficient 'encapsulation' of the compound to be delivered intracellularly, the amphiphilic system is also highly advantageous. The nucleic acid is added to *preformed* vesicles, resulting in the formation of an amphiphile/nucleic acid complex, with which, at appropriate conditions, all added DNA becomes associated. Delivery via liposomal systems, requiring DNA-encapsulation within the aqueous compartment of the vesicles, which occurs during vesicle preparation in the presence of an aqueous DNA-containing phase, is far less efficient. This is due to a far lower association of the encapsulated compound (economical disadvantage) and, in addition, minor uptake of liposomes by cells in general (1–2% of the added liposomal dose, compared to at least 40–50% of the added synthetic amphiphile preparation). Furthermore, in the case of synthetic amphiphiles, the size of the DNA is not crucial, provided that an appropriate amount of amphiphilic vesicles is incubated with the nucleic acid. This indicates that the volume of the encapsulated space, as for liposomes, is not a governing parameter in DNA-synthetic amphiphile association and that classical encapsulation of contents is not the mechanism of association. In fact, it is possible that in the process of assembly of the DNA/amphiphile complex, the dynamic polymorphic properties allow for an arrangement of the amphiphile molecules according to a nonlamellar network [24], such an arrangement being dictated by the molecular morphology of the DNA. A molecular organization of this type is consistent with the microscopic appearance of the complex as rod-like structures (Ref. [113]; see below).

Much of the transfection work involving synthetic amphiphiles has been carried out with the trimethylammonium amphiphile, DOTMA. As noted, the amphiphile is usually mixed with DOPE at a 1:1 molar ratio. At these conditions, the amphiphile stabilizes the lamellar phase of DOPE which, in isolation, readily adopts the hexagonal HII phase. However, the presence of DOPE (and hence expression of the lipid's polymorphic properties) is not crucial, since, in general, transfection can be obtained with equal efficiency when a DNA vector is introduced via DOTMA alone. However, the presence of PC, as a substitute for PE, commonly inhibits the efficiency of transfection [105,114]. Possibly, this inhibitory effect can be attributed to the fact that PC is more strongly hydrated than PE, hydration acting as a repulsion force between lipid/nucleic acid complexes and cell membrane (see also below). In addition, it is also possible that PC stabilizes the lamellar structure of the amphiphile, which may well be relevant for assembly of the DNA/amphiphile complex and/or intracellular delivery. Regarding the mechanism by which amphiphilic carriers mediate delivery, claims have been made that suggest an involvement of membrane fusion in intracellular introduction of the nucleotides [105,115]. Also endocytosis has been suggested [109,116].

With regard to the latter proposal it should be taken into account that the size of the amphiphile/DNA complex can be quite substantial (up to about 500 nm, Ref. [105,114] compared to the size of endocytic vesicles (approx. 100–150 nm; Ref. [117,118]). In spite of the fusogenic properties of amphiphiles per se, as described above, evidence to support such a mechanism for intracellular delivery of DNA is virtually lacking. Nevertheless, with erythrocytes, amphiphiles like DDP readily interact and the interaction leads to lipid dilution to an extent that is several fold higher than that observed with 'fusogenic' phospholipid vesicles, such as PS [119]. Given the state of hydration of the headgroups of amphiphiles (see previous section), electrostatic interaction between the amphiphile and target membrane may suffice for triggering 'spontaneous' merging, analogously to such asymmetric interactions between amphiphilic vesicles (DOTMA/DOPE) and anionic phospholipid vesicles (previous section).

Instructive for unraveling the mechanism of the introduction process, is a study in which it was observed that a cyclic cationic amphipathic peptide, gramicidin S may also serve the purpose of DNA carrier [114]. In this case, the presence of DOPE and in particular, its ability to display polymorphic properties were absolutely crucial to obtain transfection. Gramicidin is a membrane binding peptide with a positive charge, displaying amphipathic properties and which is capable of permeabilizing membranes [120,121]. Gramicidin A, a gramicidin of which the effect on membranes has been studied in great detail, is known for its ability to favor the L α to HII transition in PE-containing membranes [122].

Since lysomotrophic agents did not affect the level of transfection, endocytosis was excluded as the mechanism by which the DNA/gramicidin/DOPE complex gained intracellular access. This would imply that a direct translocation across the plasma membrane represents the pathway of entry for the complexed DNA. In this context, DOTMA/DOPE vesicles per se or complexed with DNA, as well as DDP vesicles, can give rise to hemolysis of erythrocytes, or release of lactate dehydrogenase from cultured cells, a cytoplasmic marker enzyme (unpublished observations). This indicates that synthetic amphiphiles, like gramicidin S also display the capacity to permeabilize cellular membranes. Similar destabilizing properties of synthetic amphiphiles have been reported for DEBDA[OH], a cationic amphiphile [73]. In this context, when the DNA/amphiphile ratio exceeds a critical value, the transfection efficiency decreases [21,105,109] and overall, a net positive charge is needed for cellular interaction. This observation suggests that when all available amphiphile molecules become mobilized in the packaging of DNA, the disruptive potency toward the target membrane is reduced to an extent that precludes the translocation of the nucleic acid across the membrane, either the plasma membrane or, after endocytosis, the endocytic membrane. By the same token, an excess of DNA may sterically interfere with

complex-membrane interaction. Indeed, at the conditions of excess DNA, the hemolytic properties of the complex are equally reduced (unpublished). Concerning the efficiency of interaction of the complex with the cell surface it is also relevant to point out here that the presence of serum strongly inhibits the transfection efficiency. In the past, it has been noted that serum also interferes with liposome-cell interaction, caused by adsorption of serum proteins on the liposomal surface [123]. It would appear that serum factors similarly 'neutralize' the interaction of amphiphiles with the cell surface. Interestingly, although optimal association of the DOTMA/DOPE/DNA complex with the cell surface is reached within 30 min, the cells need to be further incubated (in the presence of the complex) for at least 4 hours to obtain efficient transfection, whereas an incubation of only 10 min suffices to obtain this goal for DNA complexed with gramicidin S/DOPE [114]. Although this difference in kinetics of transfection need not be in conflict with a similarity in entry mechanism, i.e. a pore-mediated phenomenon, it is evident that additional mechanistic parameters, yet to be identified, are involved. Among others, such parameters may include the rate of 'dissociation' of the nucleic acid from its carrier. In addition, cellular factors may be relevant as well, since not every cell is equally susceptible to transfection while some cell lines are susceptible to transfection with lipofectin but not with gramicidin S [114]. Another prerequisite for synthetic amphiphiles in order to function as carrier for DNA appears to be the need for fluid membranes at 37°C [21]. Whether fluidity is crucial for cellular delivery or for preparing the complex of nucleic acid and synthetic amphiphile or both, is unclear. In addition, as hinted above, questions can be raised as to the extent to which *vesicular* properties are relevant to the mechanism of delivery. In fact, addition of DNA to PC/cholesterol vesicles, containing the cationic amphiphile DEBDA, resulted in lipid mixing, indicating the occurrence of nucleic acid-induced lipid reorganization [73]. Gershon et al., [113] have claimed that DNA, as a polyvalent anion, causes membrane fusion (determined by lipid mixing) of cationic DOTMA/DOPE vesicles, followed by a DNA structural phase modulation, involving an amphiphile-induced collapse of the DNA structure upon charge neutralization. Depending on the DNA-amphiphile ratio, the DNA can be shown to be fully sequestered by the amphiphile in such a way that the nucleic acid is no longer accessible to labeling with ethidium bromide or to degradation by nuclease [109,113]. Whether the cationic amphiphile and phospholipid are organized in a lamellar or non-lamellar phase (i.e., forming a nonlamellar network, using DNA as template, as referred to above) within the rodlike structure of the ultimately formed complex, remains to be determined. This study thus reveals that within the complex formed the vesicles have not maintained their original size and shape [20,124–126]. Apparently, the DNA triggers structural transformations that lead to an organization of the amphiphiles that enables the nucleic acid to

cross the plasma membrane. That this requires membrane fusion, or that the complex should be considered as DNA, encapsulated in the lipofectin vesicles remains obscure. Apart from structural, charge-induced packaging of the DNA, the amphiphile and peptide/DOPE complex add an additional parameter that facilitates the translocation. As noted above, permeabilizing properties, associated with the polymorphic properties that can be displayed by both complexes, could be part of the governing parameters.

For delivery purposes, linkage of the alkyl chains via either an ether or ester bond to the ammonium headgroup, does not appear to represent a crucial parameter. Since ether bonds are nondegradable in eukaryotic cells, it would imply that degradation of the amphiphilic complex by lipase activity is not required for intracellular release of the nucleic acid. It has been noted that an ether oxygen near the headgroup enhances the ease of flip-flop of amphiphiles, relative to such behavior for ester oxygen [127]. Therefore this propensity does not seem to play a crucial role in the delivery properties of amphiphiles.

In the context of revealing the mechanism for purposes of improving delivery efficiency, it is pertinent to bear in mind that delivery of nucleic acid and subsequent efficiency of its expression may be differently affected and hence, can be seen as distinct events in the overall process. Thus it has been reported that modulation of protein kinase C activity affected transfection efficiency [109]. Specifically, the authors suggest PKC activity to directly modulate the intracellular entry of the complex. However, the evidence presented suggests that modulation of PKC activity not likely interferes with the *mechanism* of delivery but rather with the assay used to score the transfection efficiency. Thus irrespective of the method of plasmid introduction (via calcium phosphate precipitation or via amphiphiles), activators of PKC activity were found to enhance whereas inhibitors of this activity lowered transfectability, when determined by an assay based upon measurement of chloramphenicol transferase activity [128]. In this study, cationic derivatives of cholesterol were used, containing either a tertiary or a quaternary amino headgroup. Furthermore, to obtain transfection, the presence of DOPE was an absolute requirement. The quaternary derivatives were the most efficient inhibitors of PKC activity and showed the lowest transfection efficiency. However, the same derivatives also showed a higher membrane stabilizing effect on the DOPE-containing membrane to the vesicles that were prepared from the derivative containing the tertiary amino headgroup. Hence, the distinct effect of both derivatives may, at least in part, also be related to differences in complex stability, the nucleic acid being 'released' more readily from the complex containing the tertiary derivative. Apart from the questionable role of PKC activity in the entry process per se it is also relevant to point out that the addition of one methyl group (compared to the tertiary derivative) likely causes a large change in hydration. Addition of a methyl group to PE

increases its state of hydration compared to that of PC [129], and interferes strongly with polymorphic properties of the molecules involved. It has been well documented that the state of hydration may very much affect lipid polymorphism, dehydration facilitating structural phase transitions [100,101,130]. Moreover, the cationic and anionic amphiphiles are characterized by very small headgroups, relative to the cross section of the hydrophobic part of the molecule, which readily engage in hydrogen bonding. A molecular shape factor [131] can thus be defined which predicts that, at appropriate conditions, synthetic amphiphiles display a relative strong tendency to adopt nonbilayer structures [[132], see also section IB]. It seems reasonable to assume that since membrane perturbation is involved in fusion as well as translocation, such properties would be of advantage to both processes.

3. Molecular recognition and binding

3.1. Encapsulation

Although small neutral and ionic solutes may move (almost) freely across the bilayer of vesicles formed from synthetic surfactants, larger polar compounds can be encapsulated in the aqueous compartment of the vesicle. Such compartmentalization processes are of great biological relevance and possibly have been of crucial importance in the evolution of life processes [133,134]. Efficient entrapment of solutes requires that leakage over the bilayer is greatly hampered. If this is indeed the case, fusion of vesicles will be accompanied by mixing of the aqueous contents of the bilayer systems [148] but the formation of leaky intermediates during this process may affect the efficiency of mixing. Since the entrapment of hydrophilic solutes in the aqueous vesicular compartment is an important issue in studies of vesicle fusion and in drug piloting experiments, leakage over the bilayer has been examined in some detail. For example, the highly water-soluble carboxyfluorescein (CF) has been employed as a probe to study leakage over the bilayer of DDP vesicles. This fluorescent probe was incorporated into the vesicles at self quenching concentrations. Leakage of CF over the bilayer results in dilution in the extravascular medium accompanied by relief of self quenching [135]. It was found that DDP vesicles are quite leaky compared with phospholipid vesicles. However, incorporation of 20–50 mol% of cholesterol into the bilayer decreased the leakage to a great extent. DOPC made the DDP membrane also less permeable for CF but was less effective than cholesterol (Fig. 5). Most likely, these addenda stabilize the hydrophobic core of the bilayers, thereby reducing the propensity for leakage.

Other dyes which can be encapsulated include trypan blue and the colored chromate anion. As in the case of CF, non-encapsulated dye can be separated from the vesicles

by passing over a Sephadex G75 column. The effect of additives on the permeability of the bilayer has been studied particularly in view of applications of vesicle technology which require stable vesicular dispersions and prolonged encapsulation of solutes in the aqueous compartments. For pure DDP vesicles the encapsulation efficiency of chromate ions amounts to $3.11 \text{ l} \cdot \text{mol}^{-1}$ of DDP, which is close to the values found for phospholipid MLV's [135]. Addition of the nonionic surfactant Triton X-100 led to a rapid liberation of chromate from the vesicular compartments. CF leakage across the bilayer of vesicles formed from the cationic pyridinium surfactants 1 and 2 (Fig. 6) showed that release of CF was slow for vesicles formed from 2 after sonication at 50°C [132]. A sonicated sample of 2 at 0°C gave a more rapid release of CF whereas vesicles composed of 1 and sonicated at 0°C showed a rapid loss of CF from the aqueous compartment. Interestingly, no entrapped CF was detected in vesicles prepared by the reverse-phase evaporation method.

Drug entrapment in synthetic surfactant vesicles has also been studied and comparisons with encapsulation in liposomes have been made [136]. However, for drug targeting purposes, binding of the drug in the aqueous compartment appears to be less promising because of problems due to leakage and due to effects of the polar drug on the bilayer properties. Temperature is an important parameter in determining permeability properties. Just as for liposomes, strongly increased leakage has been found for DDP vesicles around T_c . This has been rationalized by the occurrence of packing defects at the transient boundaries between macroscopic gel and liquid-crystalline domains [137] (Section 2.3). The application of mixing assays in studies of the fusion of vesicles composed of synthetic amphiphiles has generally been frustrated by practical problems involving instability and leakage of the bilayers.

3.2. Binding processes to the vesicular membrane

In Section 3 it was argued that the specific binding of fusogenic agents to the vesicular membrane is a crucial

Fig. 5. CF-leakage from vesicles of varying composition at 37°C . (O): DDP/cholesterol (90/10), (●): DDP, (□): DDP/cholesterol (80/20), (▲): DDP/cholesterol (60/40), (■): DDP/DOPC (80/20).

Fig. 6. Pyridinium surfactants 1 and 2.

event in the fusion of vesicles. Here we briefly review some general characteristics of the binding of solutes to the vesicular bilayer and how these processes affect the properties of the bilayer. Ionic, not too hydrophilic solutes often bind to the surface of the vesicular bilayer if the bilayer carries an opposite charge. The hydrophobic part of the solute may penetrate into the core of the membrane, thus leading to directional bonding. Nonpolar solutes tend to penetrate into the bilayer, thereby avoiding contact with water. Relatively little is known about the exact location of the solute and about the geometric aspects of these binding processes. It seems likely that often different binding sites may be involved. For example, ESR spin-probe experiments indicate that the nitroxide TEMPO-DNPH interacts with di-*n*-dodecyltrimethylammonium bromide (DDAB) vesicles at two binding sites: one at the bilayer surface and one in the core of the vesicular membrane [138]. Binding processes occurring in the bilayer are expected to affect the bilayer dynamics and will depend on the particular phase of the bilayer [139]. Insight into the binding process is, of course, a prerequisite for a quantitative understanding of molecular recognition processes by artificial membranes and of vesicular catalysis (section 4).

Numerous studies have been made using probe molecules which possess a medium-dependent property, usually a spectroscopic one. For example, the fluorescent probe 8-anilinonaphthalene-1-sulfonate (ANS) binds to DDAB vesicles in the region of the cationic headgroup and its fluorescence properties provide information about the surface properties of the bilayer [70]. Relevant data are shown graphically in Fig. 7 for small (average diameter 200 nm) and large (average diameter 300 nm) DDAB vesicles. Emphasis in these measurements is placed on the effect of the fusogenic dianion of dipicolinic acid (DPA^{2-}) on the properties of the DDAB bilayer-water interface (Section 3). From the wavelength of the ANS-emission maximum (λ_{max}) it may be concluded that the ANS-labeled DDAB bilayers, in the absence of DPA^{2-} , possess a polarity comparable to that of methanol which is consistent with that proposed for phospholipid bilayers [140]. The considerable blue shift upon binding of DPA^{2-} for both the small and large DDAB vesicles is indicative for a significant decrease in bilayer surface polarity. Most likely this result can be explained in term of a DPA^{2-} -induced dehydration

of the headgroups. The observed increase of the relative fluorescence intensities (Fig. 3) lends further support to this interpretation. Particularly notable is the effect of DPA^{2-} on the fluorescence polarization P for ANS (Fig. 7). In the case of the large DDAB vesicles, a sudden increase in P occurs at low (3–5 μM) concentration of DPA^{2-} . The bilayer of the small DDAB vesicles is not affected under these conditions, and a much less cooperative increase in P is only observed at higher ($\sim 12 \mu\text{M}$) concentrations of DPA^{2-} . Apparently, the first hydration layer is more tightly bound for the smaller vesicles. These bilayer curvature effects are relevant for understanding the different fusogenic behavior of small and large DDAB

vesicles [56]. Effects rather similar to those of DPA^{2-} were observed for other fusogenic agents like SO_4^- and *p*-toluenesulfonate. By contrast, nonfusogenic anions had no effect on the ANS fluorescence properties. Now the question arises whether or not the binding of DPA^{2-} to the vesicular surface effects the membrane fluidity. Therefore fluorescence depolarization was studied using the rigid, hydrophobic probe 1,6-*trans-trans-trans*-diphenyl-hexatriene-1,3,5 (DPH), intercalated in the hydrophobic core of the bilayer of small and large DDAB vesicles. It was found that the phase transition temperatures for the DPH-labeled vesicles were not changed upon incubation with DPA^{2-} . It can be concluded that the physical and

Fig. 7. Changes in ANS fluorescence properties upon interaction of DPA^{2-} with small (Δ) and large ($\circ$) ANS-labelled DDAB vesicles as a function of the concentration of DPA^{2-} : (A) wavelength of the ANS emission maximum (λ_{max}); (B) relative increase of the fluorescence intensity (I_{rel}) at the emission maximum; (C) ANS fluorescence polarization (P). Conditions: $[\text{DDAB}] = 5.5 \times 10^{-5} \text{ M}$, $[\text{ANS}] = 4.7 \times 10^{-7} \text{ M}$, $\text{pH} = 6.0$, incubation temperature 25°C .

structural alterations induced upon binding of DPA^{2-} into DDAB bilayers are restricted to changes at the vesicular surface. The situation for anionic synthetic amphiphile vesicles is completely different. It was already known that binding of Ca^{2+} to phosphatidyl serine (PS) liposomes led to a large upfield shift of the main phase transition temperature [141]. For fusion of di-*n*-alkyl phosphate vesicles Ca^{2+} is an efficient fusogenic agent (section III) and ^{31}P -NMR has been employed to study Ca^{2+} -complexation quantitatively in the concentration range employed for vesicle aggregation and fusion [142]. The phosphorus nucleus of the surfactant headgroup can also be used as an intrinsic probe for characterizing the properties of the vesicular surface by using ^{31}P -NMR spectroscopy. Two resonances were observed and the Ca^{2+} -induced upfield shifts were explained by assuming charge neutralization and dehydration of the headgroups [142]. The substantial line broadening presumably indicates a reduced mobility of the phosphate moieties. However, in contrast to the cationic vesicles, Ca^{2+} binding propagates into the hydrocarbon region of the bilayer as indicated by a substantial increase in the fluorescence polarization of membrane-bound DPH. The gel-to-liquid crystalline phase transition temperature changes from $T_c = 28^\circ\text{C}$ for DDP vesicles to a temperature above 65°C for the DDP/ Ca^{2+} complex. Apparently there is a strong Ca^{2+} -induced ordering of the alkyl chains, not only for DDP but also for a whole series of di-*n*-alkyl phosphate vesicles [55].

Hydrophobic, anionic guest recognition by macrocyclic receptors embedded in synthetic bilayer membranes has been assessed in some detail [143]. The amphiphiles contained an α -aminoacid residue interposed between the headgroup and a hydrophobic double-chain segment. Different guest-binding modes have been found. In the examples studied, the macrocycle tends to bind the guest molecules less tightly than in aqueous solution in the absence of the vesicles [144]. The presence of the membrane-bound macrocycle (steroid cyclophane) was found to affect the thermodynamics of the main phase transition of the membrane. The studies provide the first successful examples of hybrid assemblies which may be viewed of mimics as the molecule-sensor functions of receptors bound in biomembranes.

4. Catalysis by vesicles

Many biochemically relevant reactions are catalyzed by synthetic amphiphile vesicles. It appears reasonable to

assume that the vesicular surface provides a microenvironment for chemical transformations which is somewhat akin to that at the active site of enzymes. The reacting systems sense the presence of the polar or ionic headgroups, the counterions, and part of the hydrophobic alkyl chains. The Gibbs energy of the initial state and transition state of the reaction is affected by these interactions, leading to a change in reaction rate. Substrate binding is governed by noncovalent interactions due to these functionalities and if the overall Gibbs energy associated with these interactions is increased upon going to the activated complex, the local medium effect leads to a rate acceleration. However, depending on the polarity of the substrate, the reaction may also occur in the aqueous compartment of the vesicle (highly aqueous medium) or in the core of the membrane (hydrocarbon-like medium). It has been observed quite generally that the catalytic efficiency for vesicles is higher than for micelles formed from structurally related surfactants. Two effects dominate the catalysis. For unimolecular reactions, it is the local medium at the binding site(s) for the substrate that may accelerate the reaction. A popular and biologically important mimic to probe this effect is the first-order decarboxylation of 6-nitrobenz-isoxazole-3-carboxylate (6-NBIC; Fig. 8) [145,146]. The catalysis probably mainly arises from the partial dehydration of the carboxylate moiety in the initial state. Interestingly, for a series of cationic vesicle-forming amphiphiles, two isokinetic relations have been found, one above and one below the main phase transition temperature [139]. Presumably, the different thermal fluctuations of the lamellar surfaces may account for this result.

For bimolecular reactions, a second effect comes into play: the increased concentration of both membrane-bound substrates at the vesicular surface or in the core of the membrane. This entropy effect (compartmentalization) is often dominating over the medium effect. A good example is provided by the thiolysis of *p*-nitrophenyl octanoate by *n*-heptylthiol in the presence of DODAC vesicles [147]. The kinetics are more complicated than for unimolecular processes, but the pseudophase ion exchange (PPIE) model [148] is often successful in the kinetic analysis despite some questionable assumptions. Interestingly, the kinetic analysis for several reactions has shown that the actual second-order rate constant for the bimolecular reaction in the bilayer is decreased relative to that in water. Nevertheless, the reaction shows efficient catalysis since both bilayer-bound substrates react in a relatively small reaction volume compared to bulk aqueous solution.

Fig. 8. Unimolecular decarboxylation of 6-NBIC.

Enantioselective catalysis by vesicles has received much attention [149]. The surfactant molecules usually contain α -amino acid structural units and enantioselectivities up to 10^2 have been observed in hydrolytic processes [150]. Regioselective catalysis has also been studied an elegant example involving the chlorination and bromination of hydrophobically modified aromatic ethers in the presence of vesicles composed of hydrolysable surfactants [151]. The ratio of the ortho- and para-substituted reaction products varies with changing incorporation of the substrate into the bilayer. The possibility to hydrolyse the surfactant after the catalytic process may aid to work-up of the reaction mixture. Biochemically more relevant, however, is a study in which a cholesterylmetalloporphyrin has been intercalated into a synthetic biomembrane. Using this approach, regioselective epoxidation of polyunsaturated sterols and fatty acids at the double bond closest to the hydrophobic terminus of the substrate could be achieved.

Particularly interesting is Murakami's work in which vesicles formed from α -amino acid functionalized surfactants have been employed as mimics for the chemistry of Vitamine B6 and B12. These 'peptide lipids' may be cationic, anionic, zwitterionic or nonionic. Upon dispersal in water, multiwalled vesicles are formed if the number of carbon atoms in each of the two alkyl chains is equal to or greater than twelve. Single-walled vesicles obtained upon sonication are morphologically stable for an appreciable period of time. These aggregates have been successfully employed for biofunctional modelling of Vitamin B₆- and Vitamin B₁₂-dependent enzymes. Hereto hydrophobic coenzyme analogs having Vitamine B6- and Vitamine B12 activities have been bound noncovalently in the core of the vesicles. In these micro-environments the enzyme mimics display specific catalytic functions characteristic for the vitamine-dependent enzymes with substantial turn-over capabilities. This elegant and extensive work has been summarized in a review [152] but further developments continue to appear [153–155].

Finally, we briefly mention a few examples of the extensive work on vesicle-forming functionalized surfactants. The surfactant is carrying a catalytic group near the headgroup that participates in covalent bond making and breaking during the reaction. Large rate enhancements can be obtained [156,157], but the problems involved in achieving a large turn-over are far from trivial.

5. Summary and perspectives

In the present review, we have summarized a substantial body of evidence that shows how vesicles formed by synthetic amphiphiles can be used successfully to mimic aspects of the complex chemistry of cellular membranes. It appears that these types of vesicles are at least as useful as vesicles prepared from phospholipids. In fact, they offer

important advantages such as relatively easy structural variation through organic synthesis. The self-organization of synthetic amphiphiles in lamellar phases is discussed with particular attention for those aspects which are of direct relevance for understanding the physical and chemical behavior of biomembranes. Apart from molecular recognition and binding processes as well as catalytic effects, most emphasis has been placed on bilayer fusion. Efficient and fast fusion has been observed for both anionic and cationic amphiphile vesicles in the presence of proper fusogenic agents. The important finding that by selecting appropriate conditions, purely asymmetric fusion can be realized, has immediate consequences for attempts to employ synthetic amphiphiles in transfection and drug targetting experiments. The first results, using different cell lines, are promising and it is anticipated that attractive delivery systems based upon synthetic amphiphiles, will emerge in the not too far future.

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