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Review

Guanidinium group: A versatile moiety inducing transport and multicompartmentalization in complementary membranes

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Abstract

Guanidinium groups present in peptides and dendritic polymers induce their efficient transport through liposomal and cell membranes. Transmembrane crossing of these polymers is affected by their structural features and is critically dependent on the number of guanidinium groups present. Furthermore, the interaction of the guanidinium groups with phosphate groups, both located on liposomal surfaces, triggers a series of processes involving a reorganization of the self-assembled lipids and inducing the formation of multicompartment systems. These observations consistent throughout a diversity of interacting complementary liposomes, support a hypothesis that molecular recognition of liposomes induces the formation of multicompartment structures.

Keywords: Guanidinium; Membrane transport; Multicompartmentalization; Molecular recognition; Dendrimer

Contents

1. Introduction

The role of molecular transporters [1–4] and in particular of drug transporters [5–7] in enhancing membrane transport is an issue of great scientific and practical interest and has recently been addressed employing either cells or model liposomal systems. In both cases the role of an appended guanidinium group, which is the strongest organic base (pK_a 13.65) [8], is crucial due to its interaction with the phosphate or other anionic moieties on the liposomal or cell surface. This is the first stage in the process of molecular transporting in which strong binding between the interacting parties is achieved due to combined hydrogen-bonding and electrostatic forces (Fig. 1). In this connection, it should be noted that strong binding of guanidinium to acidic groups is further amplified as the organization of the interacting substrate is enhanced i.e. as one proceeds from isotropic conditions to liposomal and finally to macroscopic interfaces [9] (Fig. 2). These results clearly indicate that molecular recognition is more effective at interfaces and this binding enhancement is taken advantage of in the liposomal interactions discussed in this review.

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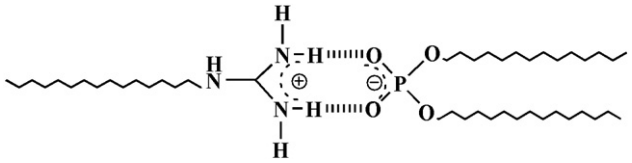

Fig. 1. Complex formed between guanidinium and phosphate groups through combined electrostatic forces and bidentate hydrogen-bonding.

The idea of extending the application of molecular transporters to guanidinylated poly(propylene imine) dendrimers [10] or other dendritic polymers has stemmed from the analogous application of arginine-rich peptides [1,2,11] which also bear a multiplicity of guanidinium moieties. These peptides proved to exhibit enhanced translocation properties, due to the presence of an appropriate number of guanidinium groups. On the other hand, guanidinylated poly(propylene imine) dendrimers having the possibility to bear a varying number of guanidinium moieties also exhibit the so-called adaptive solubility behaviour [12], rendering the dendrimer either hydrophobic or hydrophilic depending on the environment. This is irrespective of the anion effect which tunes hydrophobicity in peptides [13,14]. In the present case, following the binding of the guanidinium to the phosphate groups, guanidinylated dendrimers lie in close proximity to the membrane while they simultaneously adopt a more hydrophobic conformation. This is due to the fact that the guanidinylated derivatives bound to the liposomal surface become less polar through charge neutralization, and therefore more prone to enter the hydrophobic bilayer. In addition to charge neutralization, the adaptive solubility behaviour of dendrimers, i.e. "chameleon behaviour", as for the first time coined by Paleos et al. [12] begins to function, changing conformation and exposing their hydrophobic interior to the bilayer medium. Due to these processes the guanidinylated derivatives become appreciably hydrophobic and consequently appropriate for transport through the hydrophobic liposomal membrane.

In addition it has recently been established that interaction of guanidinium and phosphate groups, located on the surfaces of complementary liposomes, results in the formation of giant liposomes incorporating inside their interior smaller liposomes [15]. These structures are reminiscent of the multicompartment character of eukaryote cells, hypothesized to result from a symbiotic association of prokaryotes, which have analogous structures to unilamellar liposomes [16]. This so-called Serial Endosymbiosis Theory of Eukaryotic Evolution is supported by studies on mitochondria and chloroplasts which are considered to be evolved from prokaryotes (bacteria) living in large cells [17–19]. Thus, it is worth exploring this phenomenon associated with molecular recognition of complementary liposomes and formation of multicompartment systems. It seems that the role of lipids is crucial, self-assembling and organizing in membranes which may lead in the formation of either single compartment prokaryotes or multicompartment eukaryote cells [20–22].

In fact, it has to be noted that due to the organization of lipids into liposomal bilayers, the well-established organizational [9,23,24] and multivalent effects [25,26] are exercised during liposome interaction and adhesion. These effects are beneficial in obtaining enhanced initial liposomal or cell binding as previously mentioned and established by the work of Kunitake et al. [9,24]. The presence of recognizable groups in close proximity, at the relatively small external liposomal or cell surface, induces effective association between the interacting parties.

In this review we will address two processes, both of which are triggered by the presence of guanidinium moiety in appro-

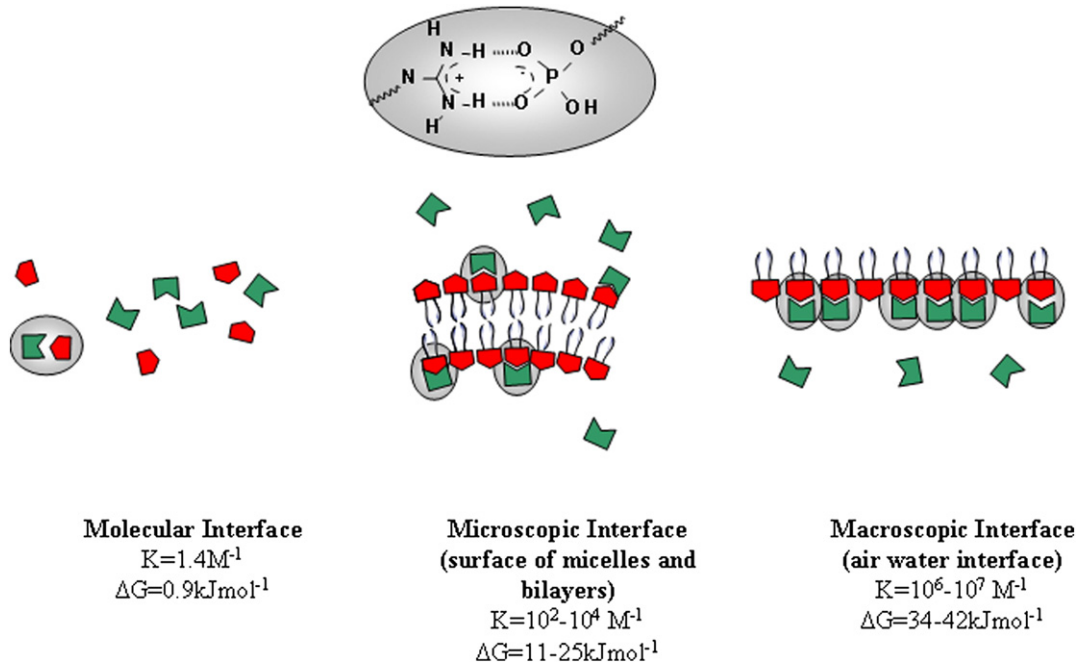

Fig. 2. Binding constants of guanidinium and phosphate groups at isotropic and other organized interfaces.

Table 1
Structure of the most extensively studied cell-penetrating peptides

Cell-penetrating peptides	Amino acid sequence
Tat_{49-57}	RKKRRQRRR
Penetratin	RQIKIWFQNRRMKWKK
Transportan	GWTLNSAGYLLKINLKALAALAKKIL

priate molecules, i.e.: A. Transport of arginine based oligopeptides and guanidinylated dendrimers through liposomal and cell membranes and B. Investigation of multicompartmentalization, resulting from the interaction of complementary liposomes, functionalized with phosphate and guanidinium groups respectively. These strategies may probably shed some light to the extremely complicated process of cell evolution. The experimental indications discussed below together with the proposed working hypothesis may constitute a starting point for addressing this issue. On the other hand they certainly lead to the explanation of practical contemporary problems, such as the processes involved in the interaction of liposomal drug delivery systems with cells [27,28].

2. Transport of arginine based peptides through liposomal and cell membranes

Drug delivery employing macromolecules has been a research challenge since they initially have to overcome the cell membrane barrier, which is impermeable to polar molecules due to its hydrophobic interior. Several natural proteins are nevertheless able to translocate across the cell membranes, with the most prominent being Tat_{49-57}, Penetratin and Transportan [29] (Table 1). These peptides have extensively been studied as carrier molecules for the delivery of bioactive compounds to either cells or liposomes. The latter represent a simple, yet efficient model for studying cell membrane transport of water-soluble molecules. In both cases interaction of the peptides led to sufficient internalization. When liposomes with different lipid composition were employed, internalization of the peptides was favored for negatively charged lipids incorporated into the liposomal bilayer [30,31].

The common feature of these peptides is that they are primarily comprised of basic amino acids i.e. L-arginine and/or L-lysine, indicating that there is a relationship between peptides with high basic amino acid residue content and their ability to translocate across model liposomal or cell membranes. Further investigations proved that the translocation properties of these peptides are directly associated with the presence of L-arginine residues. In the case of Tat peptide, replacement of L-arginine residues with less basic amino acids such as L-lysine, L-ornithine, or L-histidine has led to reduced translocation ability [32]. On the contrary replacement of all non-L-arginine residues with L-arginine resulted in a several-fold enhancement of peptide internalization [33]. In order to assess the effect of the L-arginine content on cellular uptake, studies were carried out by Futaki et al. [34] employing a series of L-arginine homopolymers bearing 4 to 16 residues. Throughout these experiments, the appropriate number of L-arginine moieties for optimum uptake was found to be between 7 and 9, since these oligomers exhibited enhanced cell accumulation, with L-arginine nonamer (R9) being 20-fold more efficient than Tat peptide. These derivatives were localized on the cytosol and the nucleus whereas oligomers with 4–6 L-arginine residues were not internalized in the cells. However oligomers with more than 10 L-arginines were mostly localized on the cell membrane. The same results were obtained by Wender et al. [33] concerning the cellular uptake of L-arginine oligomers bearing 5, 6, 7, 8 and 9 L-arginine residues.

The translocational ability of L-arginine monomer was investigated to relate to the results regarding synthetic poly-L-arginines. Membrane transport of L-arginine was studied employing sodium dihexadecyl phosphate (DHPNa) small unilamellar liposomes; its transport proved to be dependent on the organization of their lipid bilayer [35], while transport of poly-L-arginine was studied employing small unilamellar liposomes consisting of phosphatidylcholine (PC), cholesterol (Chol) and DHPNa. Moreover, fluorescence quenching experiments employing FITC-labeled poly-L-arginine demonstrated that translocation of the polymer took place at low guanidinium/phosphate molar ratios and when the liposomal bilayer is in the liquid crystalline phase [36].

In order to determine the structural requirements for cellular uptake of short arginine-rich peptides, Wender et al. [37] examined a series of L-arginine heptamers. For this purpose, aminocaproic acid (aca) was introduced as a spacer between the L-arginine residues at different structural modes as tabulated [37]. The results showed the importance of spacing for cellular

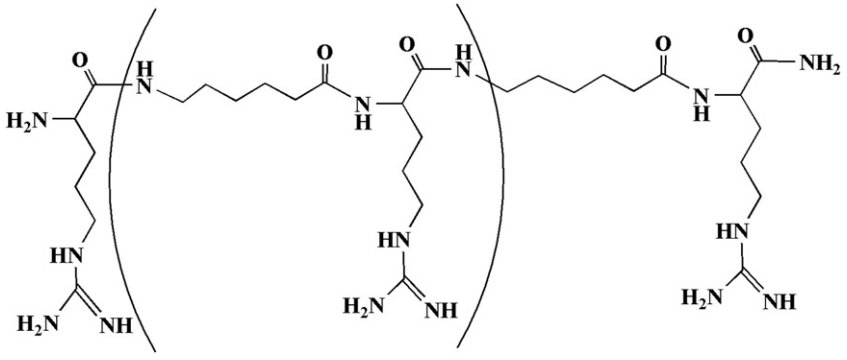

Fig. 3. Structure of $R(acaR)_5acaR$ peptide.

uptake, with the most efficient derivative being R(acaR)$_5$acaR (Fig. 3). The increased distance between the L-arginine residues resulted in increased conformational freedom of the peptide backbone. This seems to be necessary for all L-arginine residues to interact effectively with oppositely charged moieties of the membrane.

Once the importance of L-arginine was established, research was focused on the relation between the guanidinium group and the penetrating ability. Remarkably, guanidinium-rich synthetic molecules such as oligopeptoids [33] and oligocarbamates [38] (Fig. 4) demonstrated comparable or even higher translocation ability with respect to L-arginine nonamer (R9). The parameters affecting the ability of these compounds to cross cell membrane were the number of the guanidinium groups along with the distance between the guanidinium moiety and the oligomer backbone. Regarding the number of the guanidinium groups, nonamers were again the most effective. Interestingly, cellular uptake was improved when the number of alkyl spacer between the guanidinium head group and the backbone was increased from 3 (in the case of L-arginine) to 4 or 6, indicating the importance of sterical freedom of the side chains which is essential for all the guanidinium groups to interact with the cell membrane.

A possible mechanistic pathway of cell internalization has been proposed involving the formation of a non-polar complex [1,13] between the positively charged guanidinium groups and the negatively charged species present at the external surface of the cell membrane. The complex formed has the ability to cross the hydrophobic interior of the cell membrane and be effectively internalized. Initial studies [13,39] were carried out involving the partitioning of oligoarginine/polyarginine in biphasic polar/non-polar solutions. Specifically, when the highly hydrophilic guanidinium-rich transporters were introduced to a biphasic solution, i.e. water/chloroform, they were exclusively partitioned in the aqueous phase. However, addition of an amphiphilic anion, like phosphatidyl glycerol (PG) or a fatty acid or sodium dodecyl sulphate (SDS) in the organic layer resulted in almost complete partition of the guanidinylated transporter in chloroform. On the contrary hydrophilic polyanions like heparin could not mediate phase transfer of the oligoarginine transporters into the organic layer. Interestingly, when the non-polar complexes formed by the interaction of oligoarginines and amphiphilic anions were dissolved in chloroform, the presence of hydrophilic polyanions like heparin in the aqueous layer resulted in a reverse phase transfer of the transporter to the aqueous phase through counter ion exchange [13,14,39].

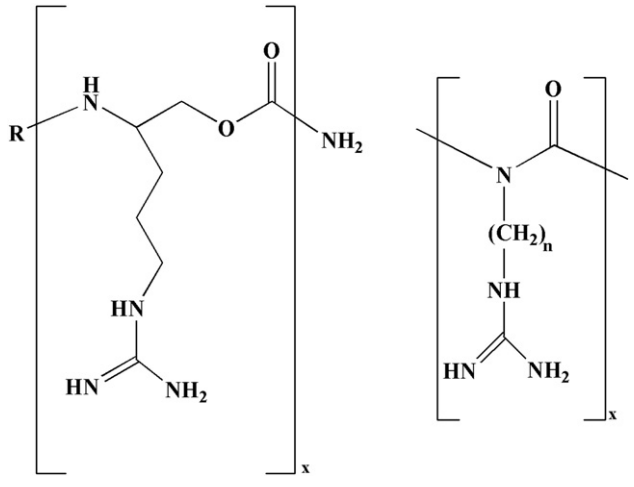

Fig. 4. Structure of cell-penetrating molecules: oligocarbamates (left) and oligopeptoids (right) with x=7–9 and n=3, 4, 6.

Advancing a step forward, liposomes were employed as a more elaborate system for elucidating the translocation mechanism. When large unilamellar liposomes consisting of phosphatidylcholine (PC) were employed, oligoarginine/polyarginine could not translocate across the lipid bilayer. However translocation was successful when anionic lipids, i.e. phosphatidylglycerol (PG) were incorporated in the lipid bilayer in a 1:1 molar ratio with respect to PC. The significance of the counter ion for the formation of the non-polar complex and its subsequent transport across the lipid bilayer was also investigated employing carboxyfluorescein (CF)-loaded liposomes composed of PC [14]. As already mentioned, these liposomes cannot mediate oligoarginine/polyarginine translocation. The presence of molecules that could form a non-polar complex i.e. pyrene butyrate or coronene butyrate, in the bulk aqueous phase resulted in an effective transport of the complex across the lipid bilayer [40]. The oligoarginine/polyarginine was subsequently interacted with the entrapped self-quenched carboxyfluorescein molecules and the resulting non-polar complex was able to cross the liposomal bilayer. Efflux of CF from the liposomal interior to the bulk aqueous phase was therefore observed by fluorescence spectroscopy.

All these experiments indicate the significance of a non-polar complex formation between oligoarginine/polyarginine and amphiphilic anions for effective transport to the bulk chloroform layer or across lipid bilayers. The formation of the non-polar complex is due to the effective interaction of the guanidinium group of the transporter with the phosphate or carboxylate groups of the amphiphilic molecules through combined electrostatic interaction and bidentate hydrogen-bonding [1,41]. This behaviour was not observed in the case of oligolysines or oligoornithines since monodentate hydrogen bonds are formed.

Charge neutralization, however, with membrane constituents cannot be the only driving force for cellular entry. The presence of a transmembrane potential, based on the intracellular and extracellular K^+ concentration gradient, was considered to have a crucial role on the accumulation of guanidinium-rich transporters. This hypothesis was set forth by Wender et al. [41] based on experiments of cellular uptake of L-arginine octamer (R8), where the membrane potential was decreased close to zero by incubating cells with an isotonic buffer (K^+PBS). Uptake of R8 was reduced up to 90% at all concentrations with respect to the uptake observed when the cells were incubated with PBS. The same result was obtained when the cells were pretreated with a pore-forming peptide i.e. Gramicidin A, which also decreases the membrane potential. On the contrary, hyperpolarizaton of the cell membrane with valinomysin, an antibiotic that selectively shuttles potassium ions across the membrane, resulted in a significant increase of the transporter internalization. Throughout these experiments it became clear that charge neutralization of the guanidinium groups of

the transporter due to non-polar complex formation is necessary for effective internalization; however the portion of the internalized complex is also directly associated with the transmembrane potential.

The structural characteristics of Tat peptide and synthetic guanidinium-rich molecular transporters have implied their use as transporter molecules for the delivery of bioactive compounds such as drugs (Taxol, Cyclosporin A), proteins (Luciferin), plasmid DNA [42] and even nanoparticles [43,44] such as magnetic nanoparticles or liposomes. In one case, modification of L-arginine heptamer with myristoyl chloride was reported [45] to facilitate delivery across the brain-blood barrier. Small molecules delivery requires the presence of an appropriate spacer group between the transporter and the drug which can be cleaved intracellularly under certain conditions in order to achieve sufficient drug accumulation. Such transporter-drug conjugates have been reported bearing either a cysteine-disulfide bond [46] or a hydrolysable ester [47] or a pH-sensitive linker [48] for Luciferin, Taxol and Cyclosporin A conjugates, respectively.

Recent studies have revealed that incorporation of Tat or oligoarginine-modified lipids into liposomes can lead to enhanced cellular uptake of these liposomal formulations or their protein complexes. It has been reported by Torchilin et al. [44] that Tat-modified liposomes with a diameter of 200 nm with the Tat-peptide located either on the liposomal surface or at the distal end of a poly(ethylene glycol) spacer, were able to transport through cell membranes. Effective uptake of these liposomal formulations by cells required the presence of 500 transporter molecules per liposome bound on their surface. In the case of oligoarginine-modified liposomes, the number of L-arginine residues was crucial in order to achieve effective cell internalization of free liposomal formulations and of liposome/protein complexes [49]. Thus, for liposomes modified with oligoarginines bearing 4, 6, 8, and 10 residues, the shorter oligoarginine led to more effective cellular uptake of the protein free liposomes, while for the intracellular delivery of liposome/protein (BSA, β-galactosidase and immunoglobulin G) complexes, liposomes modified with oligoarginines with 4 and 10 residues gave the best results. Since the guanidinium group enhances cell penetrating properties, guanidinylated polymers which can convey high drug payloads can be promising candidates for enhanced cell internalization along with effective drug delivery.

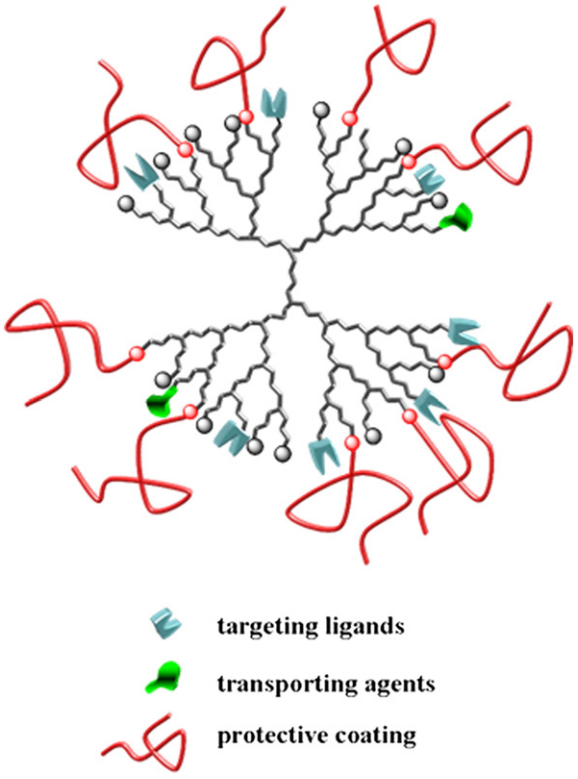

Fig. 5. Schematic representation of a multifunctional dendrimer.

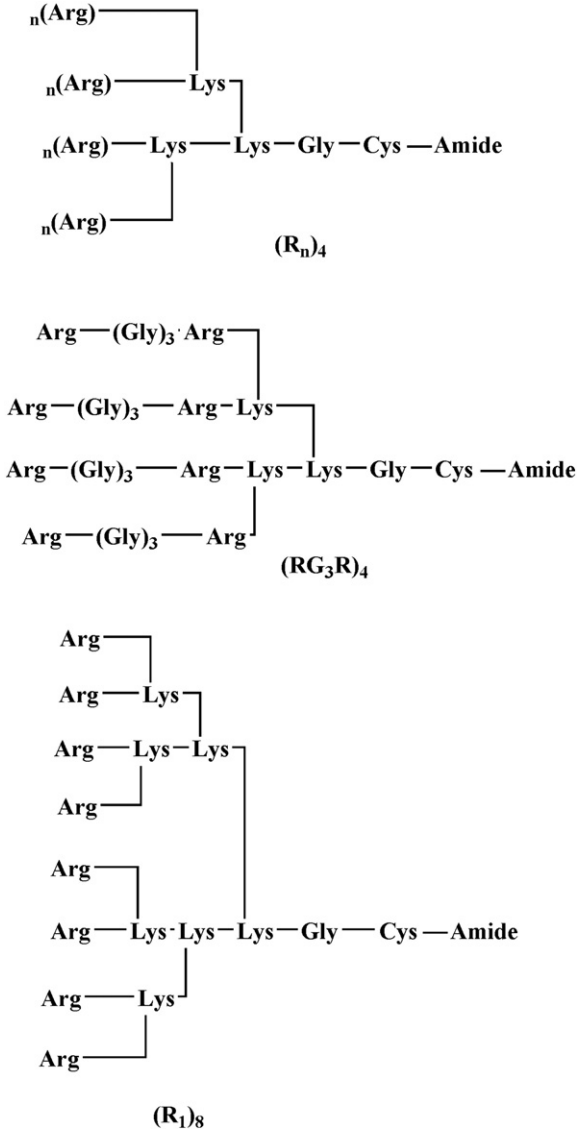

Fig. 6. Structures of branched-chain arginine-rich peptides.

3. Transport of guanidinylated dendrimers through liposomal and cell membranes

Dendritic polymers have already been studied and used as vectors for delivering bioactive compounds into cells [50–53]. The advantages of these systems originate from their nanometer-

sized architecture which induces the formation of nanocavities, the environment of which determines their solubilizing or encapsulating properties, while the external groups primarily characterize their solubility and chemical behaviour. Due to the presence of several terminal groups at their surface, a diversity of functional groups can be introduced leading to the so-called multifunctional dendrimers (Fig. 5), bearing targeting ligands, transporting agents and protective coating.

Since the role of the guanidinium group in inducing cell penetrating properties has been established, the effect of molecular architecture on molecular transporters translocation ability was further investigated. Thus, dendritic polymers, already applied as drug delivery systems and conveniently subjected to functionalization could be appropriate systems for investigating molecular transporting properties following their functionalization.

The effect of the dendritic architecture was investigated by Futaki et al. [54]. Thus dendritic molecules with varying number of L-arginine residues (from 4 to 24) were synthesized and their translocation ability was assessed (Fig. 6). The results demonstrated accumulation of these dendritic molecules into cells with the most effective being the derivative bearing 8 arginine moieties. As the number of arginine residues was either decreased or increased, the cellular uptake efficiency was reduced. Interestingly, the introduction of a spacer bearing 3 glycine moieties on the dendrimeric surface rendered the derivative with 24 arginine moieties an efficient transporter. It therefore became evident that the characteristics which render the linear guanidinium oligomers effective transporters are also applicable on dendritic derivatives. It is hence clear that the number of the guanidinium groups and not the molecular architecture of the transporter is the predominant factor enhancing internalization.

In order to elucidate the effect of branching on cellular uptake, Wender et al. [55], prepared a series of dendritic molecules bearing 8 guanidinium groups on the external surface (Fig. 7). The flexibility of the derivatives was adjusted by the introduction of various chain length spacers between the branching points. The more flexible spacing between the branching points i.e. hexyl or propyl led to derivatives exhibiting enhanced cell internalization. It is interesting to note that these dendritic compounds surpassed the most effective linear analogue, i.e. R9.

In order to investigate the translocational properties of commercially available dendrimers such as poly(propylene imine) dendrimers (DAB), a series of different generation of guanidinylated dendrimeric derivatives was prepared and their ability to cross model liposomal membranes consisting of phosphatidylcholine (PC), cholesterol (Chol) and dihexadecyl phosphate (DHP), at PC:Chol:DHP 19:9.5:1 molar ratio, was assessed [12]. DHP molecules bear the phosphate groups necessary for the interaction with the complementary guanidinium groups of the dendrimeric derivatives.

The effect of the degree of surface guanidinylation on translocation properties was evaluated employing a fourth generation poly(propylene imine) dendrimer with 32 surface amino groups (DAB32). Five dendrimeric derivatives were prepared bearing 6, 12, 18, 26 and 32 guanidinium groups. Partially guanidinylated dendrimers were quantitatively interacted with propylene oxide for replacing the toxic amino groups in order to fit for *in vitro* and *in vivo* experiments. Their ability to cross the liposomal bilayer was studied employing FITC-labeled dendrimeric derivatives which were allowed to interact with small unilamellar liposomes. The results demonstrated the enhanced translocation ability of the derivatives bearing 6, 12 and 18 guanidinium groups. These derivatives possessed the necessary number of guanidinium groups to achieve effective binding with the phosphate groups of the lipids present in the bilayer, as well as an appropriate balance between hydrophilic and hydrophobic surface groups in order to form non-polar complex able to penetrate the liposomal bilayer. This was further confirmed by the fact that the non-guanidinylated as well as highly guanidinylated derivatives had no translocational ability. Complete binding and subsequent formation of the non-polar complex are not favored for the highly guanidinylated dendrimeric derivatives.

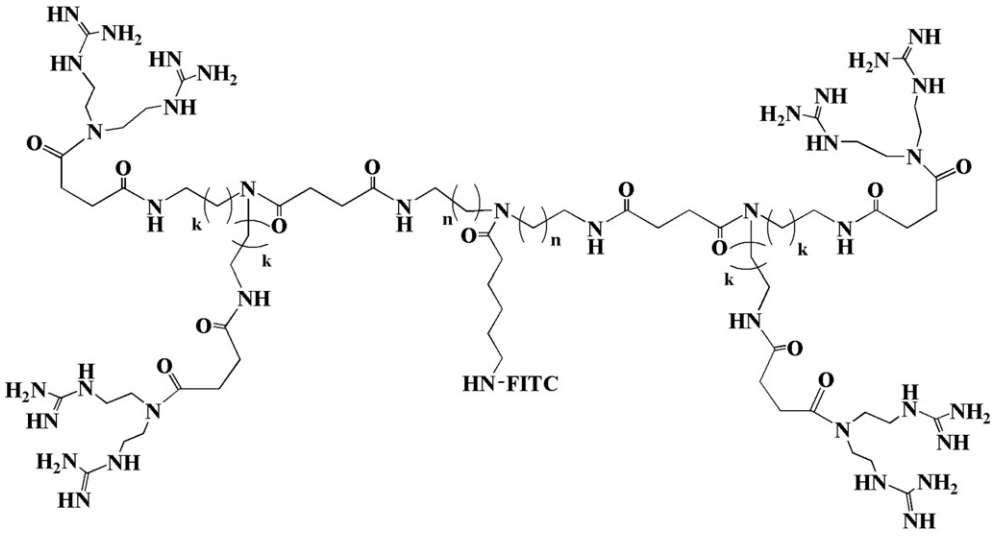

Fig. 7. Structure of dendritic molecules bearing 8 guanidinium groups at the external surface with varying chain lengths between the branching points.

The dependence of the ability to cross liposomal membranes on dendrimer generation and surface functionalization was also studied. Specifically, third and fourth generation poly(propylene imine) dendrimers (DAB16 and DAB32 respectively) with the same degree of surface guanidinylation were compared. Initially, the surface groups of dendrimers were partially acetylated while the remaining amino groups were subsequently guanidinylated, leading to derivatives possessing an appropriate balance between hydrophilic and hydrophobic groups. Furthermore, in order to investigate the effect of the spacer on the dendrimers ability to cross the liposomal bilayer, fully functionalized dendrimeric derivatives bearing a short spacer between the guanidinium group and the surface amino group were prepared (Fig. 8). When acetylated and guanidinylated dendrimers were employed, the appropriate proportion of hydrophilic and hydrophobic groups eliminated the effects attributed to dendrimers size as far as their ability to cross the lipid bilayer of small unilamellar liposomes is concerned. For the derivatives bearing a short spacer, only the third generation dendrimeric derivative showed efficient translocation. In this case the relatively small size of the third generation dendrimeric derivative along with the spacer flexibility secured the formation of the appropriate non-polar complex which subsequently crossed the liposomal bilayer. Conversely the spacer flexibility could not compensate with the increased size of the fourth generation dendrimeric derivative, leading to insufficient charge neutralization and therefore no translocational ability [56].

From these results it became evident that several structural characteristics affecting the penetrability of the linear analogues also apply to the dendritic architectures. Specifically, the degree of guanidinylation remained a crucial structural feature, and it was found that weak-to-medium guanidinylated dendrimers translocated across the liposomal bilayer while highly guanidinylated analogues exhibited reduced penetrability. Moreover, the appropriate balance between hydrophilic and hydrophobic groups is significant since the interaction of the guanidinium with the phosphate group leads, due to the spherical structure of dendrimers, to partial charge neutralization but certainly reduced hydrophilicity. Thus enhanced hydrophobicity, introduced by the presence of hydrophobic groups on the dendrimeric surface, is certainly an essential property for allowing guanidinylated dendritic polymers to cross the lipid bilayer. It has to be noted that the adaptive solubility behaviour exhibited by dendritic polymers is not only attributed to anion neutralization [12–14] or hydrophobic groups introduction on dendrimers surface [56] but it is also an intrinsic property of the dendritic polymers themselves. Specifically depending on the polarity of the medium, dendritic polymers, through conformational change, expose either their polar or their non-polar portion and therefore become soluble in solvents of differing polarity [13].

Based on the above results the following mechanism (Fig. 9) on the transport of guanidinylated dendrimers to liposomal membranes can be proposed [12]; the process may be assumed to simulate transport analogous to that in biological cells. According to this mechanism, guanidinium groups first adhere to liposomal membrane following their interaction with the negatively charged phosphate group. Strong binding is induced by multivalent effects [25,26,57]. Due to charge neutralization of the adhered guanidinylated dendrimers they become less polar and therefore susceptible to enter the hydrophobic bilayer. Synergistically to charge neutralization the adaptive solubility behaviour ("chameleon behaviour") of the dendrimers starts functioning, rendering the dendritic polymers even more hydrophobic and consequently appropriate to be transported through the hydrophobic liposomal membrane. This is in agreement with the described experimental

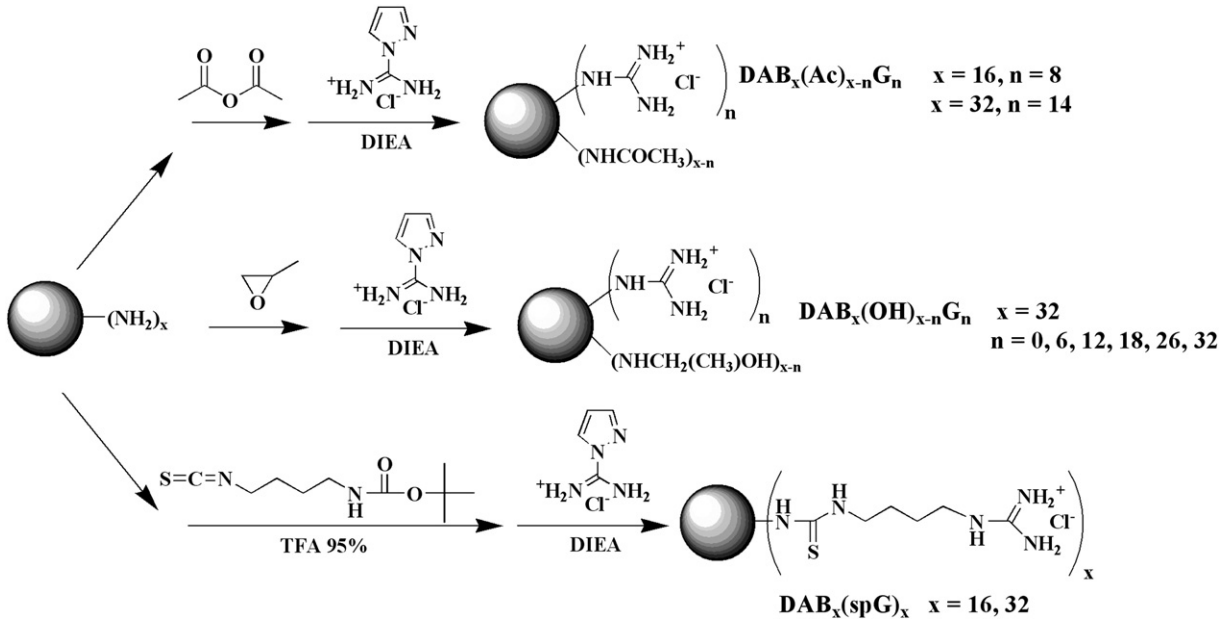

Fig. 8. Reaction scheme for the preparation of functional DAB dendrimeric derivatives.

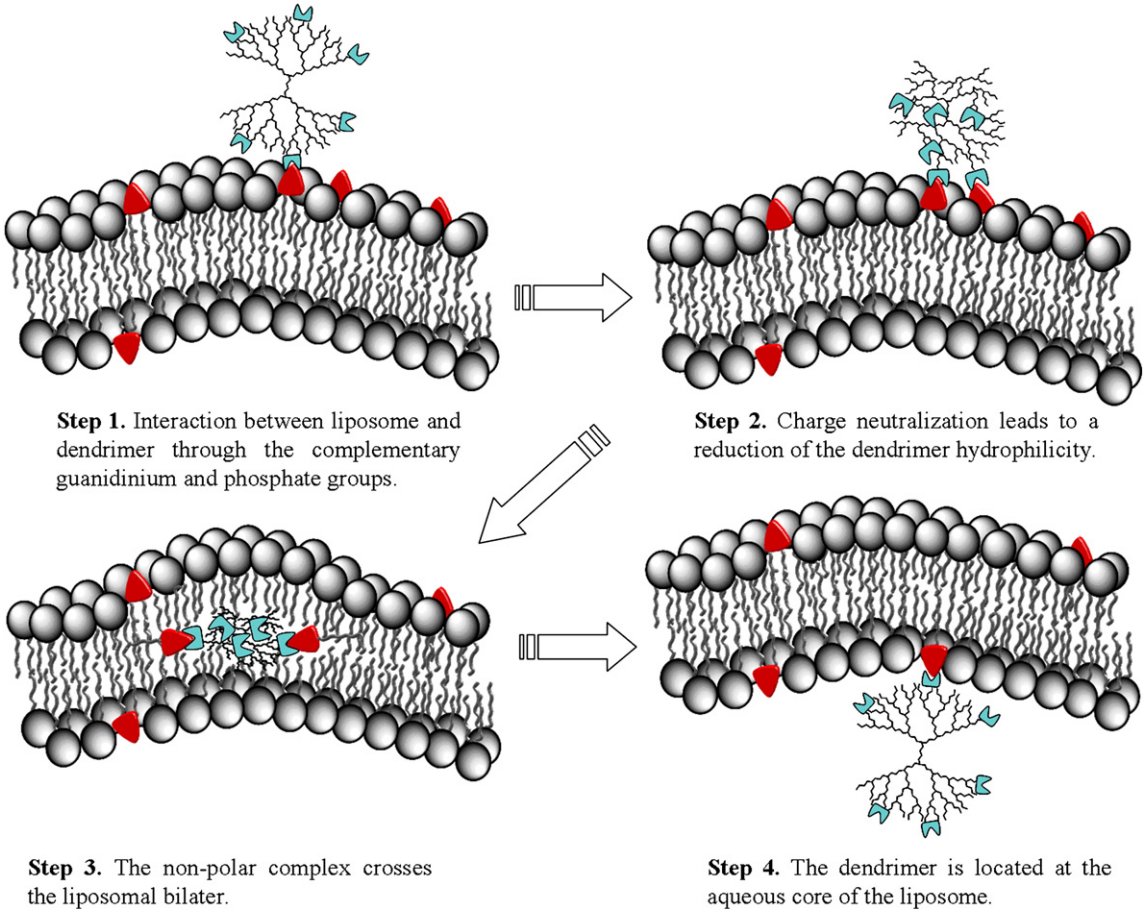

Fig. 9. Proposed mechanism for dendrimer translocation across model liposomal membranes.

results according to which weakly to medium guanidinylated dendrimers are transported through the liposomal membrane and are solubilized in their aqueous core.

4. Multicompartmentalization induced by the interaction of guanidinylated liposomes with their complementary analogues

Organization of liposomal lipids [9], multivalency [25,26] and combined electrostatic forces with bidentate hydrogen-bonding between guanidinium and phosphate groups induces strong binding of complementary liposomes. The subject of molecular recognition of complementary liposomes has been thoroughly investigated by Lehn et al. [58], Paleos et al. [15,59–63] and reviewed [64].

Following a strong liposomal adhesion by which the complementary liposomes are simply conjoined with retention of their inner compartments they subsequently merge by fusion sharing a common inner compartment [65,66]. A sequence of processes gives rise to the mixing of the aqueous content of liposomes, with or without leakage or rupture of the fused vesicles. The compartments may remain intact or products of liposomal fusion may be obtained, further reacting and leading to the formation of multicompartment systems. In the examples that will be discussed we will primarily highlight the property of multicompartmentalization since this is the one leading to multicompartment systems formation.

The term "multicompartmental aggregate" was coined by Zasadzinski's group [67] for aggregates originating from molecular recognition of liposomes mediated by biotin–streptavidin complex formation. A multi-stage procedure was proposed, in which the key step was molecular recognition between complementary moieties whereas the final step involved an unrolling of cochleate cylinders (multilamellar lipid tubules) bound to liposomal aggregates, which led to the encapsulation of the latter within the bilayer of the cylinders. In this manner, multicompartment systems were formed. In connection with multicompartment systems formation, it should be mentioned that encapsulation of liposomes and colloidal particles was also achieved by a procedure based on cochleate cylinders with elimination of the specific recognition steps. Thus, in a recent work [68], when Ca^{2+} was added to liposomes of dioleoylphosphatidylserine (DOPS), fusion resulted in the formation of multilamellar cochleate cylinders of anionic bilayers. In an optional step, small liposomes or colloidal particles were subsequently added to the dispersion of these cylinders. Subsequently, complexation of Ca^{2+} upon addition of EDTA results in an unrolling of the cylinders and reclosure of the bilayers. This process leads to the formation of micrometer-sized DOPS giant liposomes which encapsulate the added small liposomes or colloidal particles. It should be noted

that although the processes for multicompartmentalization differ, the presence of cochleate cylinders is the critical step for the formation of multicompartment systems.

Triggered by the above results on multicompartment systems formation, induced by complicated molecular recognition processes [67], we were led to carefully observe the nature and structure of aggregates obtained by molecular recognition experiments between liposomes [15,59–63]. This aimed at investigating whether the aggregates obtained exhibit multicompartment structures and, if this is the case, to uncover factors triggering or inducing their formation.

For molecular recognition between liposomes to occur their membrane is usually tailored by incorporating cholesterol, protective coating and indispensably recognizable groups. A model of such an interacting complementary pair of liposomes is shown in (Fig. 10) while examples on molecular recognition between liposomes are discussed below.

Unilamellar mixed liposomes of about 100 nm diameter were prepared [59] consisting basically of phosphatidylcholine (PC), and cholesterol (Chol). For accomplishing molecular recognition of these liposomes, one type of liposomes incorporated di-*n*-hexadecylphosphate (DHP), while the other 1-[4-(dihexadecylcarbamoyl)butyl]guanidinium *p*-toluenesulfonate (DBG). The strong binding between DHP and DBG moieties has allowed experiments to be performed at low molar ratios of these lipids relative to PC (molar ratio PC/DGB and PC/DHP = 19:1). Cholesterol was also incorporated in the liposomal membrane at various concentrations ranging from 10% to 50% molar with respect to PC simulating the cell membrane composition. The so-prepared liposomes interacted spontaneously providing large aggregates which were possible to observe by optical microscopy. As concluded from their dimensions, fusion follows initial adhesion leading to large liposomes, which in certain cases encapsulate smaller ones. These structures exhibit a kind of compartmentalization, which very primitively mimic the compartments present in biological cells (Fig. 11).

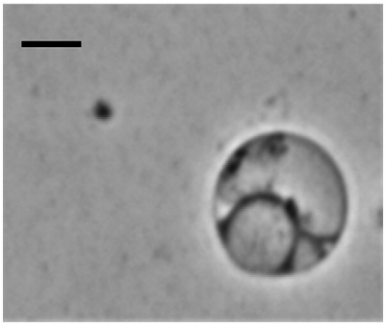

Fig. 11. Phase-contrast optical microscopy image of a liposomal aggregate obtained after mixing complementary liposomes. The bar in the upper-left corner indicates 5 μm.

A significant outcome of this work, indirectly related to compartmentalization is that cholesterol incorporated in these liposomes appreciably enhances their molecular recognition effectiveness. The molecular recognition enhancement, which was observed at cholesterol concentrations ranging from 10% to 50% molar with respect to PC, was attributed to the structural features of lipid–cholesterol bilayers. This finding was rationalized on the basis of cholesterol's effect upon the molecular order of the lipid bilayer according to a widely accepted phase diagram [69–73]. According to this diagram, at the temperature range in which these experiments were performed and at cholesterol concentrations higher than 25% molar with respect to PC, the liquid-ordered phase is formed. The fact that this phase is fluid, from the viewpoint of lateral disorder and diffusion, is significant for the molecular mobility of the recognizable molecules. On the other hand, since the recognizable lipids are incorporated at a low molar ratio (1:19), their presence does not appreciably perturb the molecular organization of the PC–Chol bilayer and therefore, the interacting moieties encounter the previously mentioned organized environment permitting their mobility. Apparently, molecular organization combined with

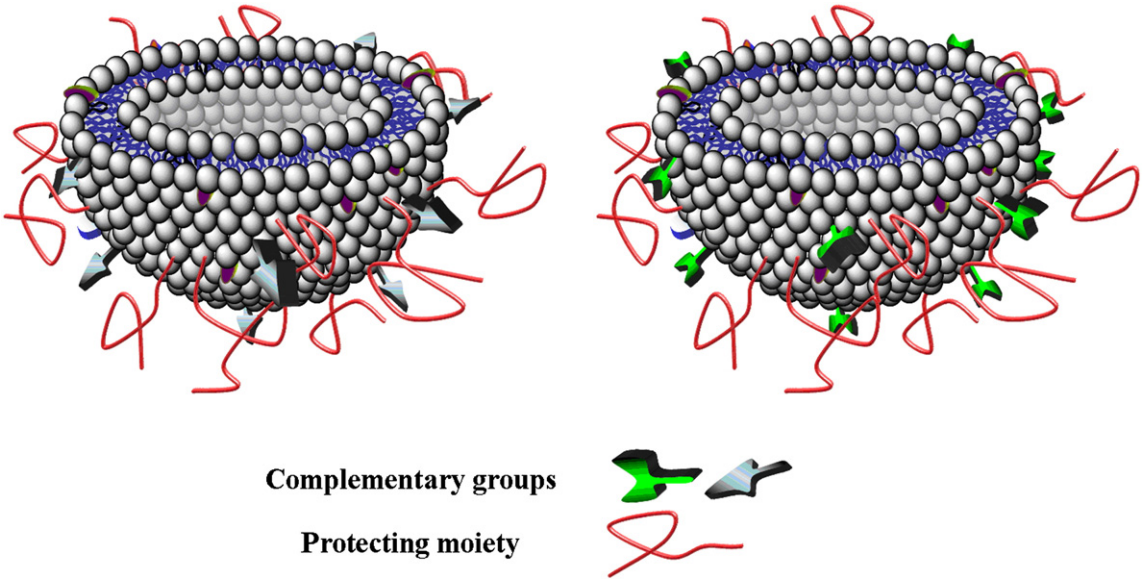

Fig. 10. Schematic representation of a multifunctional complementary pair of liposomes.

fluid lateral mobility of the recognizable lipids in the liquid-ordered phase, results in a more enhanced association of the liposomes. The enhancing role of cholesterol in liposomal association, observed by microscopic and light scattering studies, was quantitatively established [59] by Isothermal Titration Microcalorimetry (ITC). The role of cholesterol in liposomal recognition is also evident from the reaction rates, which were determined by (ITC) experiments. Assuming single-exponential kinetics, the reaction rates (k) become approximately 4 times faster in the presence of cholesterol.

Extending the previous study, the inhibitory role of the protective polyethylene glycol (PEG) coating on molecular recognition was investigated [15,60], employing liposomes based on hydrogenated PC and cholesterol and incorporating recognizable moieties on their surface. One type of liposomes incorporated DHP, whereas their complementary counterparts contained either octadecylguanidine hydrochloride (ODG), *N*-[3-(octadecylamino)propyl]guanidine hydrochloride (ODPG), or *N*-[3-(dioctadecylamino)propyl]guanidine hydrochloride (DOPG) (Fig. 12). With the application of the two latter guanidinylated lipids, the role of the propylene spacer on the recognition effectiveness of liposomes was assessed. Due to the presence of the spacer, the guanidinium group protrudes from the liposomal interface and therefore its interaction with the complementary phosphate group is facilitated. PEG coating of molecular weight 5000 was introduced on the surface of liposomes through the incorporation of a varying number of PEGylated cholesterol molecules in the liposomal membrane. This is a convenient strategy for the attachment of the PEG at the liposomal surface in which the cholesterol moiety is effectively anchored inside the liposomal membrane. Following mixing of the complementary liposomes multicompartment aggregates were observed (Fig. 13) by phase-contrast microscopy while their size was confirmed by dynamic light scattering (DLS).

The fusion of complementary liposomes occurs under a non-leaking process involving lipid mixing as it was established by calcein entrapment and Resonance Energy Transfer experiments [15]. The thermodynamic parameters correlate with the processes of aggregation and fusion. Thus the interactions of non-PEGylated liposomes consistently involve exothermic processes of much higher enthalpic content compared to the PEGylated counterparts. Thus, for the pairs [PC:Chol:ODPG]–[PC:Chol:DHP] and [PC:Chol:ODG]–[PC:Chol:DHP], the ΔH values are −5.7 and −3.0 Kcal/mol respectively, while for the PEGylated liposomes (5% PEG relative to cholesterol) the ΔH values are −3.8 and −1.1 Kcal/mol respectively.

Modeling the interaction of drug-loaded liposomes with cells the interaction of loaded unilamellar with multilamellar

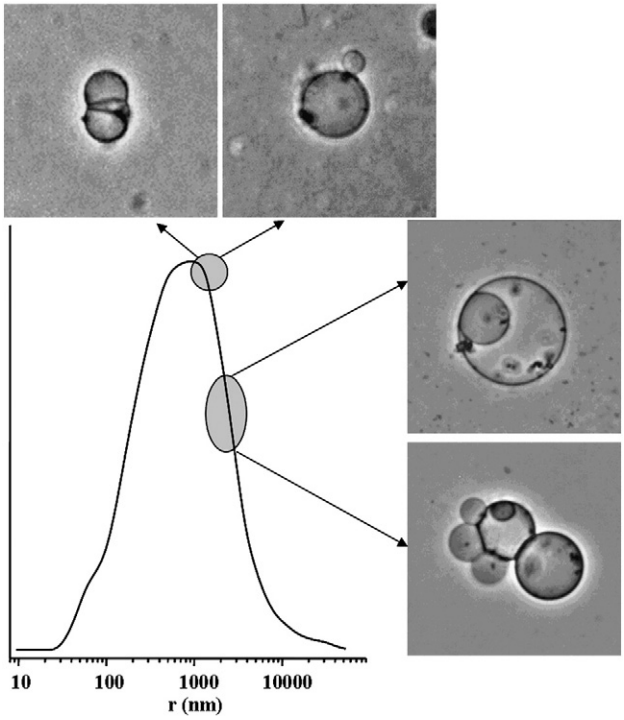

Fig. 13. Phase-contrast optical microscopy images of liposomal aggregates after mixing PEGylated complementary unilamellar liposomes. Their sizes correspond with the size distribution observed by DLS. The bar in the lower-left corner indicates 5 μm.

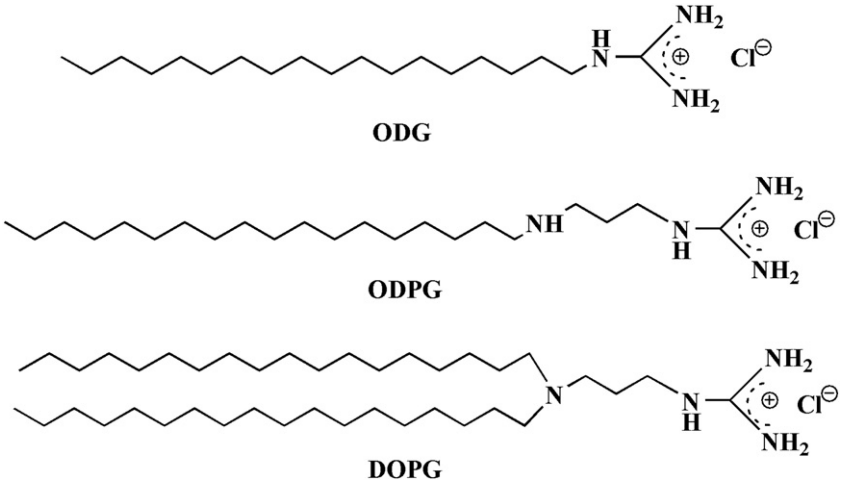

Fig. 12. Structures of guanidinylated lipids.

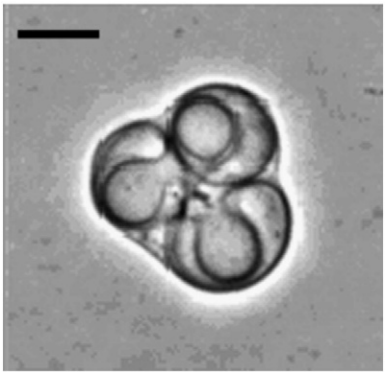

Fig. 14. Phase-contrast optical microscopy image of a liposomal aggregate after mixing samples of unilamellar and multilamellar complementary liposomes. The bar in the upper-left corner indicates 5 μm.

liposomes was investigated. The lipids used for the preparation of liposomes were the same as in the previous experiment [61]. Nevertheless, one type of liposomes of the interacting complementary pair was now multilamellar. Highlighting on optical microscopy studies, multicompartment systems were also observed (Fig. 14), which were consistent, regarding their sizes, with DLS measurements. This experiment further illustrates the hypothesis [74] that molecular recognition, induced by the interaction of surface guanidinium and phosphate groups, leads to the formation of multicompartment systems.

Focusing on multicompartment systems formation, induced by the interaction of guanidinium with phosphate groups, the following mechanisms [74] may be proposed: Fusion between liposomes is facilitated by the presence on their surface of moieties that can molecularly recognize each other i.e. complementary liposomes. In this manner liposomes of various sizes are obtained (steps I and II, Fig. 15), which are generally characterized as large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV). According to Lehn et al. [58], which investigated an analogous complementary pair of liposomes, adhesion leading to fusion does not take place between giant liposomes, while a selective LUV-GUV adhesion does occur leading to fusion (interaction step III, Fig. 14). This last step can rationalize multicompartment systems formation, according to which large liposomes are introduced in the interior of giant liposomes through an engulfation and recognition step, reminiscent of endocytosis. In the lower row of Fig. 15, the images observed with phase-contrast microscopy are shown, corresponding to the hypothesized in each step structures. It has to be noted however, that giant liposomes are in general susceptible to various transformations which are triggered either by the addition of appropriate chemicals or by temperature change, as reviewed by Menger and Angelova [75]. Aggregates, analogous to the ones presented in this review, have been prepared and a mechanism of membrane fusion was proposed [76] justifying the incorporation of a smaller liposome to larger ones.

5. Concluding remarks

The role of guanidinium groups in peptides and dendritic polymers is crucial in inducing transport through liposomal and cell membranes; this behaviour is also dependent on the structural features of the polymers. Furthermore the interaction of guanidinium groups located on the liposomal surface with phosphate groups of complementary counterparts triggers a series of processes, involving reorganization of the membranous lipids leading to the formation of multicompartment systems. These multicompartment systems were repeatedly observed even-though lipid composition of the interacting liposomes was varied. Due to the structural similarities between unilamellar liposomes and prokaryotic cells one may be led to a working hypothesis according to which multicompartmentalization in eukaryotic cells resulted in a fashion analogous to multicompartmentalization from the interaction of liposomes.

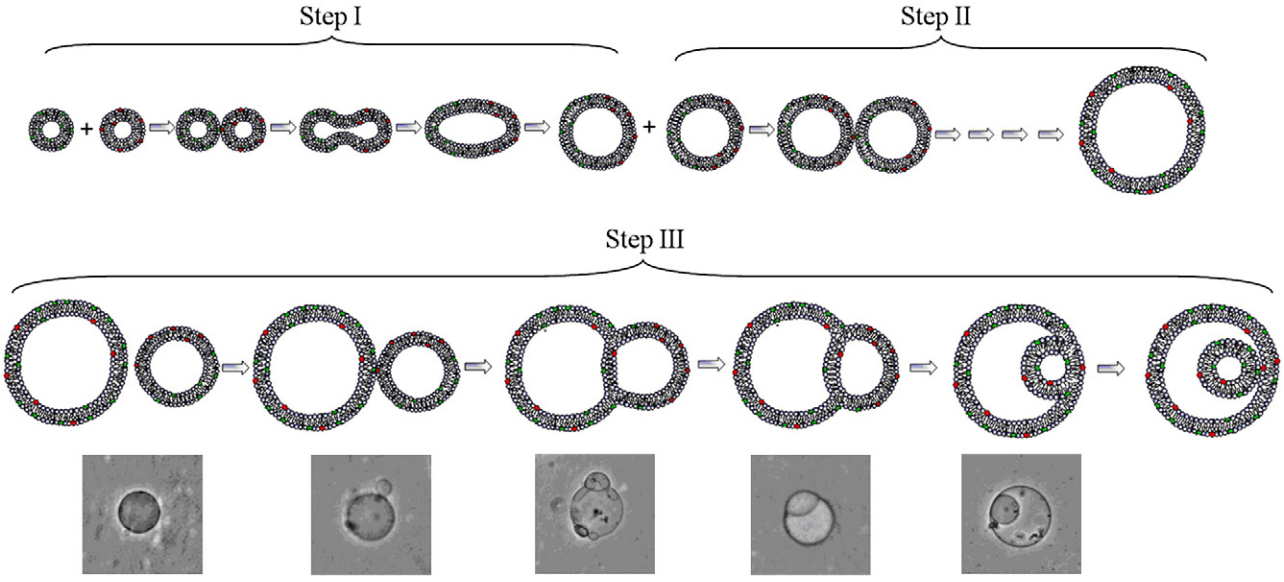

Fig. 15. Proposed mechanism presenting the steps of formation of multicompartment liposomal structures in correspondence with phase-contrast optical microscopy images.

References

[1] J.B. Rothbard, T.C. Jessop, P.A. Wender, Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells, Adv. Drug Deliv. Rev. 57 (2005) 495–504.

[2] S. Futaki, Membrane-permeable arginine-rich peptides and the translocation mechanisms, Adv. Drug Deliv. Rev. 57 (2005) 547–558.

[3] S. Futaki, S. Goto, Y. Sugiura, Membrane permeability commonly shared among arginine-rich peptides, J. Mol. Recognit. 16 (2003) 260–264.

[4] J.S. Wadia, S.F. Dowdy, Modulation of cellular function by TAT mediated transduction of full length proteins, Curr. Protein Pept. Sci. 4 (2003) 97–104.

[5] L.R. Wright, J.B. Rothbard, P.A. Wender, Guanidinium rich peptide transporters and drug delivery, Curr. Protein Pept. Sci. 4 (2003) 105–124.

[6] R.B. Kim, Transporters and drug discovery: why, when, and how, Mol. Pharmaceutics 3 (2006) 26–32.

[7] L. Zhang, J.M. Strong, W. Qiu, L.J. Lesko, S.-M. Huang, Scientific perspectives on drug transporters and their role in drug interactions, Mol. Pharmaceutics 3 (2006) 62–69.

[8] C.M. Paleos, D. Kardassi, D. Tsiourvas, Transformation of dihexadecyl phosphate vesicles to micelles using guanidinium-type counterions, Langmuir 15 (1999) 282–284 (and references sited therein).

[9] M. Onda, K. Yoshihara, H. Koyano, K. Ariga, T. Kunitake, Molecular recognition of nucleotides by the guanidinium unit at the surface of aqueous micelles and bilayers. A comparison of microscopic and macroscopic interfaces, J. Am. Chem. Soc. 118 (1996) 8524–8530.

[10] M. Najlah, A. D'Emanuele, Crossing cellular barriers using dendrimer nanotechnologies, Curr. Opin. Pharmacol. 6 (2006) 522–527.

[11] C.-H. Tung, R. Weissleder, Arginine containing peptides as delivery vectors, Adv. Drug Deliv. Rev. 55 (2003) 281–294.

[12] I. Tsogas, D. Tsiourvas, G. Nounesis, C.M. Paleos, Modeling cell membrane transport: interaction of guanidinylated poly(propylene imine) dendrimers with a liposomal membrane consisting of phosphate-based lipids, Langmuir 22 (2006) 11322–11328.

[13] N. Sakai, T. Takeuchi, S. Futaki, S. Matile, Direct observation of anion-mediated translocation of fluorescent oligoarginine carriers into and across bulk liquid and anionic bilayer membranes, ChemBioChem 6 (2005) 114–122.

[14] N. Sakai, S. Futaki, S. Matile, Anion hopping of (and on) functional oligoarginines: from chloroform to cells, Soft Matter 2 (2006) 636–641.

[15] A. Pantos, D. Tsiourvas, Z. Sideratou, C.M. Paleos, Interactions of complementary PEGylated liposomes and characterization of the resulting aggregates, Langmuir 20 (2004) 6165–6172.

[16] G.M. Cooper, The cell: a molecular approach, ASM Press, Washington DC, 2008, pp. 467–517.

[17] L. Sagan, On the origin of mitosing cells, J. Theor. Biol. 14 (1967) 255–274.

[18] L. Margulis, Origin of eukaryotic cells, Yale University Press, New Haven, 1970.

[19] L. Margulis, Symp. Symbiotic Theory of the Origin of Eukaryotic Organelles, Criteria for Proof, vol. 29, 1975, pp. 21–38.

[20] D. Segré, D. Ben-Eli, D.W. Deamer, D. Lancet, The lipid world, Orig. Life Evol. Biosph. 31 (2001) 119–145.

[21] P.A. Monnard, D.W. Deamer, Membrane self-assembly processes: steps toward the first cellular life, Anat. Rec. 268 (2002) 196–207.

[22] D.D. Lasic, Liposomes within liposomes, Nature 387 (1997) 26–27.

[23] K. Ariga, T. Kunitake, Molecular recognition at air–water and related interfaces: complementary hydrogen bonding and multisite interaction, Acc. Chem. Res. 31 (1998) 371–378.

[24] D.Y. Sasaki, K. Kurihara, T. Kunitake, Specific, multiple-point binding of ATP and AMP to a guanidinium-functionalized monolayer, J. Am. Chem. Soc. 113 (1991) 9685–9686.

[25] M. Mammen, S.-K. Choi, G.M. Whitesides, Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors, Angew. Chem., Int. Ed. Engl. 37 (1998) 2755–2794.

[26] P.I. Kitov, D.R. Bundle, On the nature of the multivalency effect: a thermodynamic model, J. Am. Chem. Soc. 125 (2003) 16271–16284.

[27] T.M. Allen, P.R. Cullis, Drug delivery systems: entering the mainstream, Science 303 (2004) 1818–1822.

[28] E.T. Kisak, B. Coldren, C.A. Evans, C. Boyer, J.A. Zasadzinski, The vesosome-A multicompartment drug delivery vehicle, Curr. Med. Chem. 11 (2004) 1241–1253.

[29] M. Zorko, Ü. Langelb, Cell-penetrating peptides: mechanism and kinetics of cargo delivery, Adv. Drug Deliv. Rev. 57 (2005) 529–545.

[30] P.E.G. Thorén, D. Persson, P. Lincoln, B. Nordén, Membrane destabilizing properties of cell-penetrating peptides, Biophys. Chem. 114 (2005) 169–179.

[31] P.E.G. Thorén, D. Persson, E.K. Esbjörner, M. Goksör, P. Lincoln, B. Nordén, Membrane binding and translocation of cell-penetrating peptides, Biochemistry 43 (2004) 3471–3489.

[32] D.J. Mitchell, D.T. Kim, L. Steinman, C.G. Fathman, J.B. Rothbard, Poly-arginine enters cells more efficiently than other polycationic homopolymers, J. Pept. Res. 56 (2000) 318–325.

[33] P.A. Wender, D.J. Mitchell, K. Pattabiraman, E.T. Pelkey, L. Steinman, J.B. Rothbard, The design, synthesis and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13003–13008.

[34] S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, Y. Sugiura, Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery, J. Biol. Chem. 276 (2001) 5836–5840.

[35] I. Tsogas, D. Tsiourvas, C.M. Paleos, S. Giatrellis, G. Nounesis, Interaction of L-arginine with dihexadecylphosphate unilamellar liposomes: the effect of the lipid phase organization, Chem. Phys. Lipids 134 (2005) 59.

[36] I. Tsogas, D. Tsiourvas, G. Nounesis, C.M. Paleos, Interaction of poly-L-arginine with dihexadecyl phosphate/phosphatidylcholine liposomes, Langmuir 21 (2005) 5997–6001.

[37] J.B. Rothbard, E. Kreider, C.L. VanDeusen, L. Wright, B.L. Wylie, P.A. Wender, Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake, J. Med. Chem. 45 (2002) 3612–3618.

[38] P.A. Wender, J.B. Rothbard, T.C. Jessop, E.L. Kreider, B.L. Wylie, Oligocarbamate molecular transporters: design, synthesis, and biological evaluation of a new class of transporters for drug delivery, J. Am. Chem. Soc. 124 (2002) 13382–13383.

[39] N. Sakai, S. Matile, Anion-mediated transfer of polyarginine across liquid and bilayer membranes, J. Am. Chem. Soc. 125 (2003) 14348–14356.

[40] F. Perret, M. Nishihara, T. Takeuchi, S. Futaki, A.N. Lazar, A.W. Coleman, N. Sakai, S. Matile, Anionic fullerenes, calixarenes, coronenes, and pyrenes as activators of oligo/polyarginines in model membranes and live cells, J. Am. Chem. Soc. 127 (2005) 1114–1115.

[41] J.B. Rothbard, T.C. Jessop, R.S. Lewis, B.A. Murray, P.A. Wender, Role of membrane potential and hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells, J. Am. Chem. Soc. 126 (2004) 9506–9507.

[42] A. Astriab-Fisher, D.S. Sergueev, M. Fisher, B.R. Shaw, R.L. Juliano, Antisense inhibition of P-glycoprotein expression using peptide–oligonucleotide conjugates, Biochem. Pharmacol. 60 (2000) 83–90.

[43] M. Lewin, N. Carlesso, C.-H. Tung, X.-W. Tang, D. Cory, D.T. Scadden, R. Weissleder, Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells, Nat. Biotechnol. 18 (2000) 410–414.

[44] V.P. Torchilin, R. Rammohan, V. Weissig, T.S. Levchenko, TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 8786–8791.

[45] W. Pham, B.-Q. Zhao, E.H. Lo, Z. Medarova, B. Rosen, A. Moore, Crossing the blood-brain barrier: a potential application of myristoylated polyarginine for in vivo neuroimaging, NeuroImage 28 (2005) 287–292.

[46] L.R. Jones, E.A. Goun, R. Shinde, J.B. Rothbard, C.H. Contag, P.A. Wender, Releasable Luciferin-transporter conjugates: tools for the real-time analysis of cellular uptake and release, J. Am. Chem. Soc. 128 (2006) 6526–6527.

[47] T.A. Kirschberg, C.L. VanDeusen, J.B. Rothbard, M. Yang, P.A. Wender, Arginine-based molecular transporters: the synthesis and chemical evaluation of releasable taxol-transporter conjugates, Org. Lett. 5 (2003) 3459–3462.

[48] J.B. Rothbard, S. Garlington, Q. Lin, T. Kirschberg, E. Kreider, P.L. McGrane1, P.A. Wender, P.A. Khavari, Conjugation of arginine oligomers

to cyclosporin A facilitates topical delivery and inhibition of inflammation, Nat. Med. 6 (2000) 1253–1257.
[49] M. Furuhata, H. Kawakami, K. Toma, Y. Hattori, Y. Maitani, Intracellular delivery of proteins in complexes with oligoarginine-modified liposomes and the effect of oligoarginine length, Bioconjug. Chem. 17 (2006) 935–942.
[50] S.-E. Stiriba, H. Frey, R. Haag, Dendritic polymers in biomedical applications: from potential to clinical use in diagnostics and therapy, Angew. Chem., Int. Ed. Engl. 41 (2002) 1329–1334.
[51] A. D' Emanuele, D. Attwood, Dendrimer–drug interactions, Adv. Drug Deliv. Rev. 57 (2005) 2147–2162.
[52] E.R. Gillies, J.M.J. Fréchet, Dendrimers and dendritic polymers in drug delivery, Drug Discov. Today 10 (2005) 35–43.
[53] C.M. Paleos, D. Tsiourvas, Z. Sideratou, Molecular engineering of dendritic polymers and their application as drug and gene delivery systems, Mol. Pharmaceutics 4 (2007) 169–188.
[54] S. Futaki, I. Nakase, T. Suzuki, Z. Youjun, Y. Sugiura, Translocation of branched-chain arginine peptides through cell membranes: flexibility in the spatial disposition of positive charges in membrane-permeable peptides, Biochemistry 41 (2002) 7925–7930.
[55] P.A. Wender, E. Kreider, E.T. Pelkey, J. Rothbard, C.L. VanDeusen, Dendrimeric molecular transporters: synthesis and evaluation of tunable polyguanidino dendrimers that facilitate cellular uptake, Org. Lett. 7 (2005) 4815–4818.
[56] I. Tsogas, Z. Sideratou, D. Tsiourvas, T.A. Theodossiou, C.M. Paleos, Interactive transport of guanidinylated poly(propylene imine)-based dendrimers through liposomal and cellular membranes, ChemBioChem. 8 (2007) 1865–1876.
[57] J.D. Badjic, A. Nelson, S.J. Cantrill, W.B. Turnbull, J.F. Stoddart, Multivalency and cooperativity in supramolecular chemistry, Acc. Chem. Res. 38 (2005) 723–732.
[58] V. Marchi-Artzner, T. Gulik-Krzywicki, M.-A. Guedeau-Boudeville, C. Gosse, J.M. Sanderson, J.-C. Dedieu, J.-M. Lehn, Selective adhesion, lipid exchange and membrane-fusion processes between vesicles of various sizes bearing complementary molecular recognition groups, Chem. Phys. Chem. 2 (2001) 367–376.
[59] Z. Sideratou, D. Tsiourvas, C.M. Paleos, A. Tsortos, G. Nounesis, Molecular recognition of complementary liposomes: the enhancing role of cholesterol, Langmuir 16 (2000) 9186–9191.
[60] A. Pantos, Z. Sideratou, C.M. Paleos, Complementary liposomes based on phosphatidylcholine: interaction effectiveness vs protective coating, J. Colloid Interface Sci. 253 (2002) 435–442.
[61] A. Pantos, D. Tsiourvas, C.M. Paleos, G. Nounesis, Enhanced drug transport from unilamellar to multilamellar liposomes induced by molecular recognition of their lipid membranes, Langmuir 21 (2005) 6696–6702.
[62] C.M. Paleos, Z. Sideratou, D. Tsiourvas, Mixed vesicles of didodecyldimethylammonium bromide with recognizable moieties at the interface, J. Phys. Chem. 100 (1996) 13898–13900.
[63] D. Tsiourvas, Z. Sideratou, A.A. Haralabakopoulos, G. Pistolis, C.M. Paleos, Molecular recognition of amphiphilic di(dodecyl)barbituric acid with long-chain alkylated adenine and thymine derivatives, J. Phys. Chem. 100 (1996) 14087–14092.
[64] C.M. Paleos, Z. Sideratou, D. Tsiourvas, Molecular recognition of complementary liposomes in modeling cell–cell recognition, ChemBioChem 2 (2001) 305–310.
[65] G. Cevc, H. Richardsen, Lipid vesicles and membrane fusion, Adv. Drug Deliv. Rev. 38 (1999) 207–232.
[66] R. Blumenthal, M.J. Clague, S.R. Durell, R.M. Epand, Membrane fusion, Chem. Rev. 103 (2003) 53–69.
[67] S.A. Walker, M.T. Kennedy, J.A. Zasadzinski, Encapsulation of bilayer vesicles by self-assembly, Nature 387 (1997) 61–64.
[68] C.C. Evans, J.A. Zasadzinski, Encapsulating vesicles and colloids from cochleate cylinders, Langmuir 19 (2003) 3109–3113.
[69] J.H. Ipsen, G. Karlström, O.G. Mouritsen, H. Wennerström, M.J. Zuckermann, Phase-equilibria in the phosphatidylcholine-cholesterol system, Biochim. Biophys. Acta 905 (1987) 162–172.
[70] J.L. Thewalt, M. Bloom, Phosphatidylcholine—cholesterol phase—diagrams, Biophys. J. 63 (1992) 1176–1181.
[71] C. Trandum, P. Westh, K. Jørgensen, O.G. Mouritsen, A thermodynamic study of the effects of cholesterol on the interaction between liposomes and ethanol, Biophys. J. 78 (2000) 2486–2492.
[72] O.G. Mouritsen, K. Jørgensen, Dynamical order and disorder in lipid bilayers, Chem. Phys. Lipids 73 (1994) 3–25.
[73] O.G. Mouritsen, K. Jørgensen, Dynamic lipid-bilayer heterogeneity — a mesoscopic vehicle for membrane-function, BioEssays 14 (1992) 129–136.
[74] C.M. Paleos, D. Tsiourvas, Interaction between complementary liposomes: a process leading to multicompartment systems formation, J. Mol. Recognit. 19 (2006) 60–67.
[75] F.M. Menger, M.I. Angelova, Giant vesicles: imitating the cytological processes of cell membranes, Acc. Chem. Res. 31 (1998) 789–797.
[76] T. Tanaka, M. Yamazaki, Membrane fusion of giant unilamellar vesicles of neutral phospholipid membranes induced by La^{+3}, Langmuir 20 (2004) 5160–5164.