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Developing and applying a drug delivery model for liposomal and dendritic multifunctional nanoparticles

Review Article

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Abbreviations: Adriamycin, (ADR); barbituric acid, (BAR); betamethasone dipropionate, (BD); betamethasone valerate, (BV); cholesterol, (CHOL); diaminobutane poly(propylene imine) dendrimer, (DAB); 5,5-didodecylbarbituric acid, (DBA); 1-(4-(dihexadecylcarbamoyl)butyl)guanidinium p-toluenesulfonate, (DBG); di-n-hexadecylphosphate, (DHP); N-[3-(dioctadecylamino)propyl] guanidine hydrochloride, (DOPG); doxorubicin, (DXR); dynamic light scattering, (DLS); giant unilamellar vesicles, (GUV); 9-hexadecyladenine, (HA); hyperbranched polyglycerol, (PG); Isothermal Titration Microcalorimetry, (ITC); large unilamellar vesicles, (LUV); monomethyl ether polyethylene glycol, (M-PEG); N-[3-(octadecylamino)propyl] guanidine hydrochloride, (ODPG); octadecylguanidine hydrochloride, (ODG); phosphatidylcholine, (PC); poly(amidoamine) dendrimer, (PAMAM); poly(propylene imine) dendrimers of the fourth generation functionalized with n guanidinium groups, (DAB-Gn); polyethylene glycol, (PEG); tamoxiphen, (TMX); 2,4,6 triaminopyrimidine, (TAP)

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Summary

This account deals with a strategy for designing multifunctional liposomes and dendritic polymers. Such nanoparticles, although quite different in size and structure, both fulfill properties that drug carriers should exhibit, i.e. specificity or targeting ability, extended time of circulation in biological fluids and ability to be transported through cell membranes. Furthermore, having developed these multifunctional liposomal and dendritic carriers, a drug delivery model is presented that employs instead of cells multilamellar liposomes, which interact with the above mentioned multifunctional carriers. This interaction should primitively mimic the processes which occur in living cells when they interact with loaded or unloaded liposomal and dendritic nanoparticles. Multifunctionality and multivalency coupled with molecular recognition between the interacting pairs render the loaded nanoparticles effective drug delivery vehicles.

I. Introduction

A significant number of bioactive molecules fail to be commercialized as drugs due to lack of tissue specificity, blood solubility, metabolic stability or bioavailability. In order to address these issues effective drug delivery systems, including liposomes and dendritic polymers (comprising of dendrimers and hyperbranched polymers) are being developed.

Liposomal and dendritic nanoparticles although quite different in size, structural features and consequently in loading properties, both are susceptible to surface functionalization by analogous strategies. In this manner, nanoparticles sharing common functional groups can be obtained. In fact, liposomes and dendritic polymers have been developed bearing ligands which target to cell receptors or protective groups that prevent their phagocytosis and prolong their circulation in biological fluids. The latter property is almost exclusively achieved by coating the surface of the carriers with polyethylene-glycol (PEG) chains applying the well-known strategy of PEGylation (Allen, 1994; Lasic and Needham, 1995; Needham and Kim, 2000; Liu et al, 2000; Gabizon et al, 2003). Another crucial parameter of effective drug delivery systems is their transport through cell membranes, which can be achieved by the introduction of

translocating agents on the surface of the nanocarriers. The application of cell penetrating peptides (Prochiantz, 2000; Futaki et al, 2003; Wright et al, 2003; Gorodetsky et al 2004; Futaki, 2005a; Kim, 2006; Maeda and Fujimoto, 2006) and specifically of arginine-rich derivatives which have been extensively studied and found to exhibit enhanced membrane translocation ability, can be employed as the basis for preparing efficient molecular transporting liposomal and dendritic nanoparticles. Hence, by employing oligo- and poly- arginine derivatives the overall molecular transporting process is facilitated; this is attributed to the presence of the guanidinium moiety which interacts with the phosphate and carboxylate groups of the cells surface (Vivès et al, 1997; Wender et al, 2000; Futaki et al, 2001, 2005b; Kirschberg et al, 2003;).

The strategy for the preparation of typical multifunctional liposomes and dendritic polymers is described in this account. These nanoparticles, **Figure 1**, differing in size and structural features in principle fulfill the above mentioned properties for drug carriers, i.e. specificity or targeting ability, long circulation in biological fluids and transport properties through cell membranes. Furthermore having developed these multifunctional liposomal and dendritic carriers, a drug delivery model is presented employing, instead of cells, multilamellar liposomes. The latter primitively mimic the processes involved in biological cells during their interaction with loaded or unloaded liposomal and dendritic nanoparticles.

II. Interaction between complementary liposomes

Conventional liposomes have long been employed as drug delivery systems for a diversity of polar and lipophilic bioactive compounds (Gregoriadis, 1995; Barenholz, 2001; Guo and Szoka, 2003). These liposomes, however, are in general unstable in biological media and do not exhibit specificity for certain cell types. These issues were satisfactorily addressed through appropriate functionalization of the liposome surface achieved by a selective choice of the lipids. Specifically, the preparation of these functional liposomes was realized by using the so-called bottom-up strategy in which functional surfactants are used as building blocks for their bilayer formation. The development of multifunctional liposomal nanoparticles should be the ultimate objective for obtaining highly effective drug delivery systems. In fact, in the early stage of these investigations, mono-functional liposomes were prepared which were not bearing all the required functional groups. Recently, a step-wise strategy was adopted for the preparation of multifunctional liposomes as will be briefly discussed below. Complementary liposomes interact mimicking in a way the recognition of cells with liposomes.

Molecular engineering of liposomal surfaces, by the introduction of functional groups having the ability to form hydrogen bonds with complementary moieties introduced to other liposomes, leads to the formation of large aggregates when this pair of liposomes is allowed to interact. During this interaction the following processes take place (Cerv and Richardsen, 1999): a. Adhesion during which the liposomes are simply conjoined but retain separate inner compartments and b. Fusion during which the liposomes merge sharing a common inner compartment. In this second process a sequence of events occurs, giving rise to the mixing of the aqueous content of liposomes with or without leakage or rupturing of the fused vesicles.

With regard to the mechanism of membrane fusion, two processes have been investigated, i.e. the conventional one involving non-lamellar fusion and that of restructuring and ultimately merging of the membrane on a less ordered basis. It has been supported that both mechanisms are energetically favoured at least for small-scale fusion. However, it has to be noted that small liposomes, i.e. with diameters of about 45nm comprising of less than 10000 molecules, do not have the sufficient number of amphiphiles to form a non-lamellar phase without disintegrating; these liposomes are however known to fuse extensively, apparently through membrane restructuring (Cerv and Richardsen, 1999). Taking into account the above mentioned associative mechanisms, processes such as the exchange of amphiphiles or the effect of liposomal size on the efficiency of interaction should also play an important role in the binding of liposomes. Bearing these considerations in mind and with the understanding that fusion of phospholipids liposomes has been used as a model for simulating biological membrane fusion, the cited examples on liposomal interactions will be discussed.

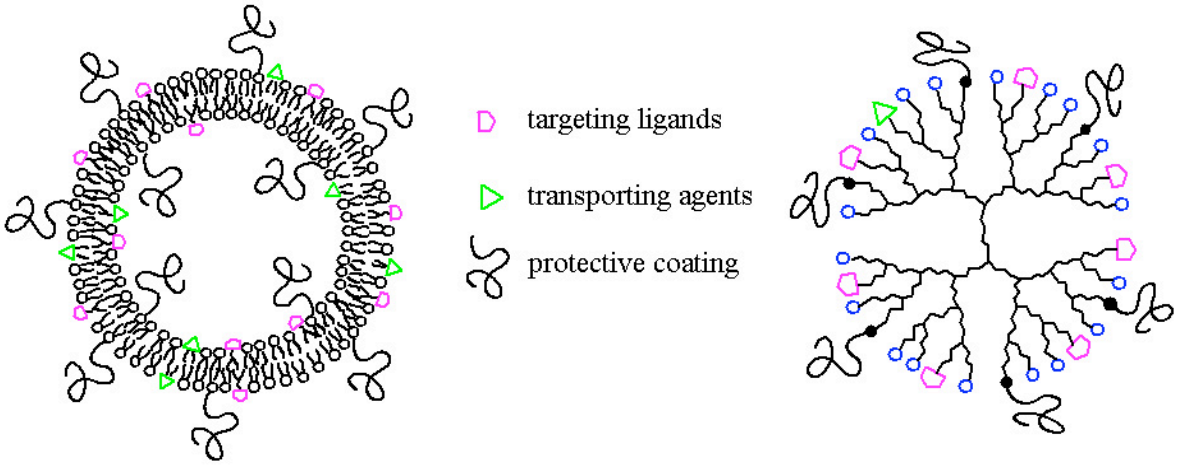


Figure 1. Schematic representation of a multifunctional liposome (left) and dendrimer (right).

As previously mentioned, the functionalization of the external surface of liposomes was achieved through the preparation of mixed liposomes. In this manner, the functional moieties on the external surface, originate from the amphiphilic components, which were incorporated into these liposomes. It is thus possible to monitor the reactivity of liposomes by changing the type and concentration of the incorporated recognizable lipid. In this context, early work (Paleos et al, 1996; Marchi-Artzner et al, 1997) triggered the interest in investigating molecular recognition between complementary liposomes. In the following years Lehn et al (Marchi-Artzner et al, 2001) further investigating their previously employed complementary pair, established the supramolecular chemistry of interacting liposomes. This pair of liposomes consisted basically of egg phosphatidylcholine also containing amphiphilic derivatives of barbituric acid (BAR) or triaminopyrimidine (TAP) (**Figure 2**), each up to 10% molar ratio. The recognition of the complementary moieties was facilitated by the insertion of a suitable spacer in between the recognizable and polar groups. The main conclusions of this study are summarized below:

a. Rapid and selective aggregation (in less than 30 s) occurs between the complementary liposomes, followed by lipid exchange (within 30 min after mixing). The lipid exchange, which takes place when the membranes are in contact, results either in fusion or, if fusion does not occur, to a redispersion of the liposomes within 17 hours.

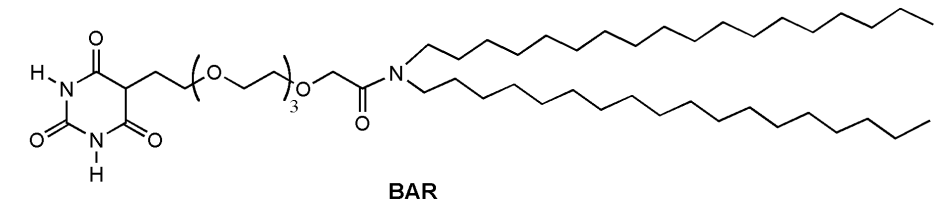
b. The aggregation process of the system under investigation can be weakened by decreasing the ionic strength, through the addition of a soluble barbituric competitor or by decreasing the concentration of the recognizable amphiphiles. The effect of ionic strength underlines the basic role of electrostatic interaction in the initial aggregation step. On the other hand, the effect of the recognizable amphiphiles supports the view according to which the recognizable system stabilizes the adhesion state.

c. The fusion process was observed by electron microscopy and remained at a low level not resulting in an intermixing of the aqueous pools of the liposomes to a significant degree. It seems that fusion resulted from the collapse of mixed triaminopyridine/barbituric acid liposomes with neighbouring liposomes.

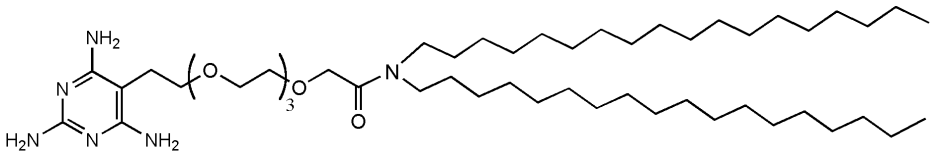
d. The size of the liposomes has a crucial effect on the recognition phenomena. Thus aggregation was not observed when giant liposomes were encountered. The interaction between recognizable groups is not sufficiently strong to establish a stable contact between giant liposomes. A rapid adhesion however occurs between complementary large and giant unilamellar liposomes.

Continuing the investigation on the reactivity of complementary liposomes (Sideratou et al, 2000), unilamellar liposomes of about 100 nm diameter were prepared consisting basically of phosphatidylcholine (PC), and cholesterol (CHOL)**.** For accomplishing molecular recognition of these liposomes, one part of them incorporated di-n-hexadecylphosphate (DHP), while the other part 1-(4-(dihexadecylcarbamoyl)butyl)guanidinium p-toluenesulfonate (DBG) as recognizable lipids (**Figure 3**). Cholesterol was incorporated in the liposomal membrane at various concentrations starting from 10% and up to 50% molar with respect to PC simulating in this way cell membrane composition. The so-prepared complementary liposomes were allowed to interact at ambient temperature. The complementary guanidinium and phosphate groups, located on liposomal surfaces, interact strongly due to electrostatic and hydrogen-bonding forces promoting, by their combined action, non-covalent bonding (Onda et al, 1996) between these liposomes (**Figure 3**). This strong interaction between DHP and DBG lipids allowed experiments to be performed at low molar ratios of these lipids relative to PC (molar ratio PC/DGB and PC/DHP=19:1). Due to molecular recognition of these liposomes, interaction did occur and large aggregates were obtained which were large enough to be observed even by optical microscopy. As concluded from their dimensions, fusion follows initial adhesion leading to large multicompartment aggregates, which in certain cases encapsulate smaller ones.

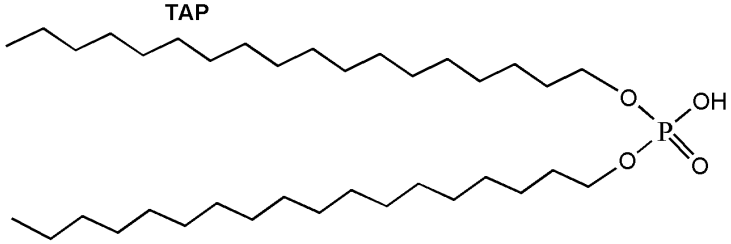
These structures, shown in **Figure 4**, exhibit a form of compartmentalization, which represents a simplistic analogue of subcellular compartments. The formation of these multicompartment systems was recently presented elsewhere (Paleos and Tsiourvas, 2006) and it is further discussed below.



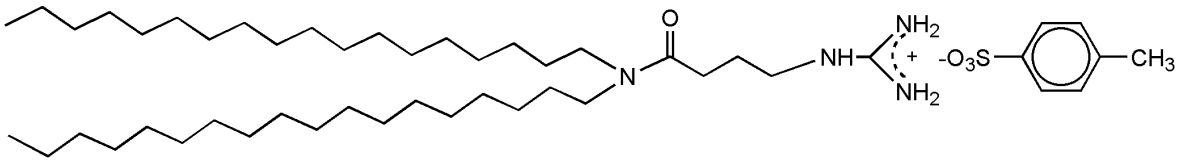
BAR



TAP



DHP



DBG

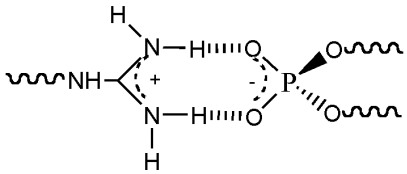


Figure 2. Chemical structures of amphiphilic derivatives of barbituric acid (BAR) and triaminopyrimidine (TAP).

Figure 3. Chemical structures of di-n-hexadecylphosphate (DHP) and 1-(4-(dihexadecylcarbamoyl)butyl) guanidinium p-toluenesulfonate (DBG) and the interaction scheme between the complementary phosphate and guanidinium groups.

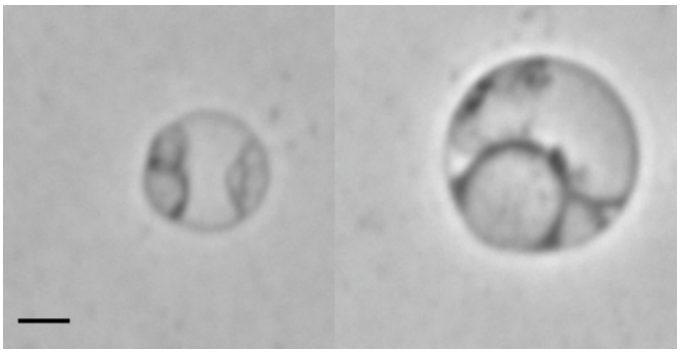


Figure 4. Multicompartment aggregates obtained following molecular recognition of complementary liposomes incorporating DHP and DBG. The bar in the lower left corner indicates 5μm.

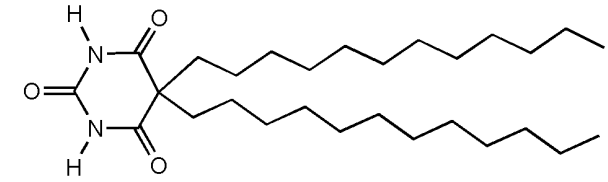
A significant, yet unexpected, outcome of this work (Sideratou et al, 2000) is that the cholesterol incorporated in these liposomes appreciably enhances their molecular recognition effectiveness. The molecular recognition

enhancement, which was observed at cholesterol concentrations ranging from 10% to 50% molar with respect to phosphatidylcholine, was attributed to the structural features of lipid-cholesterol bilayers. This finding was explained on the basis of effect of cholesterol on the molecular ordering in the lipid bilayer according to a widely accepted phase diagram (Ipsen et al, 1987; Mouritsen and Jørgensen, 1992, 1994; Thewalt and Bloom, 1992; Trandum et al, 2000). According to this diagram, within the experimental 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 allowing their mobility. Apparently, molecular organization combined with fluid lateral mobility of the recognizable lipids in the liquid–ordered phase, results in a more enhanced association of the liposomes.

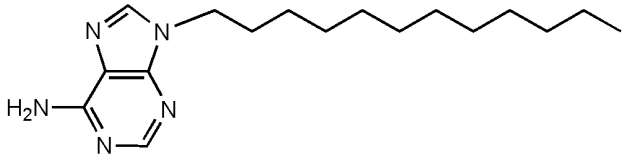
The role of encapsulated cholesterol in liposomal membrane, for enhancing liposomal association, as observed by microscopic and light scattering studies, was further established (Sideratou et al, 2000) by isothermal microcalorimetry. The heat released by the interaction of the complementary liposomes was maximum for the system [PC:CHOL:DBG]/[PC:CHOL:DHP] and negligible for all the control experiments. Thus, the binding enthalpy was found to be 1.113 kJ mol^{-1}, when cholesterol (50%) was present and 0.576 kJ mol^{-1} in its absence, i.e. in the control experiment. The role of cholesterol in liposomal recognition is also evident from the reaction rates, which were determined by Isothermal Titration Microcalorimetry (ITC) experiments. Assuming single-exponential kinetics, the reaction rates (k) become approximately 4 times faster in the presence of cholesterol. It is therefore evident that cholesterol should be incorporated in liposomes for both stabilizing their membrane and also for enhancing their association ability.

Based on the above findings on the role of cholesterol in liposomal membrane an analogous system was prepared (Sideratou et al, 2002a), in which the recognizable amphiphiles 5,5-didodecylbarbituric acid (DBA) and 9-hexadecyladenine (HA) (**Figure 5**) were incorporated in liposomes based on hydrogenated phosphatidylcholine and cholesterol in a PC:CHOL 2:1 molar ratio. Hydrogen-bonding interactions between these complementary lipid moieties (**Figure 5**) were relatively weak, and therefore the recognizable lipids were incorporated at a high molar content relative to PC (i.e. Recognizable Lipid:PC 1:4). In this manner, relatively strong binding between the liposomes was obtained and accurate determination of the thermodynamic parameters was achieved. Molecular recognition of liposomes becomes most effective at 1:1 molar ratio of the recognizable molecules. Following mixing of the complementary liposomes multicompartment aggregates were obtained, which have structural analogies to the ones previously observed. These aggregates were apparently obtained following fusion of the initially adhering liposomes under non-leaking conditions. Investigation of molecular recognition between this pair of liposomes was also investigated by ITC and one-to-one binding between the adenine and barbituric acid moieties in the lipid/water/lipid interface was observed (Sideratou et al, 2002a). The effect of cholesterol on the mechanism of binding between DBA and HA at the lipid-water interface was found to be in concord with a previous study (Sideratou et al, 2000). Thus, in analogy with the previous experiment, the presence of cholesterol rendered the binding process faster, at least at low DBA/HA molar ratios.

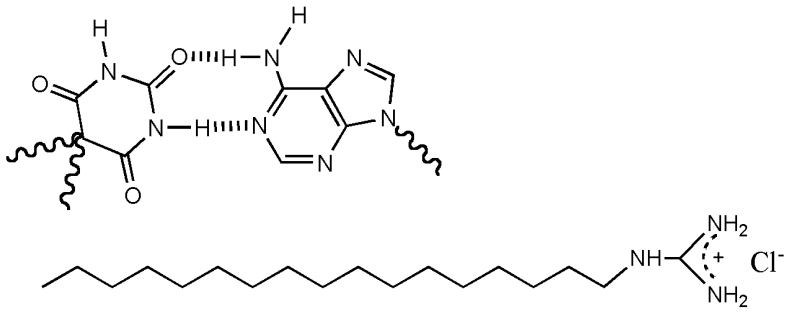
In recent studies on liposomal interactions (Pantos et al, 2002, 2004) the inhibitory role of the protective polyethylene glycol (PEG) coating on molecular recognition was investigated. In this sense, liposomes consisting of hydrogenated PC and cholesterol were prepared, incorporating recognizable moieties on their surface. One type of liposomes incorporated DHP, whereas their complementary liposomes contained either octadecylguanidine hydrochloride (ODG), or N-[3-(octadecylamino)propyl] guanidine hydrochloride (ODPG), or N-[3-(dioctadecylamino)propyl] guanidine hydrochloride (DOPG) (**Figure 6**). With the application of the two latter guanidinylated lipids, the role of the propylene spacer on the recognition effectiveness of liposomes was evaluated. Due to this spacer, the guanidinium group protrudes from the liposomal interface and therefore its probability of interaction with the complementary phosphate group is enhanced.



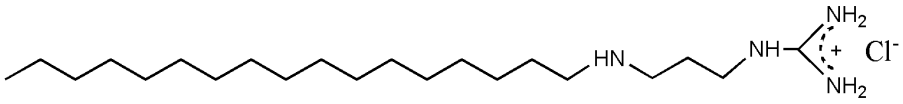
DBA



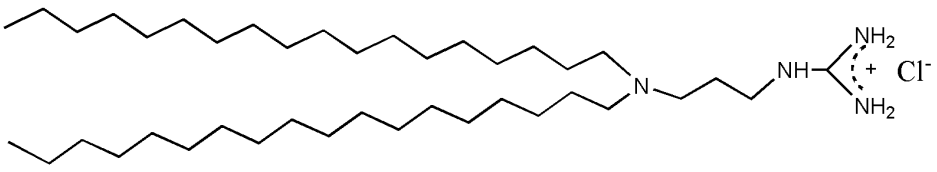
HA



ODG



ODPG



DOPG

Figure 5. Chemical structures of 5,5-didodecylbarbituric acid (DBA) and 9-hexadecyladenine (HA) and the interaction scheme between the complementary barbituric and adenine groups.

Figure 6. Chemical structures of octadecylguanidine hydrochloride (ODG), N-[3-(octadecylamino)propyl] guanidine hydrochloride (ODPG) and N-[3-(dioctadecylamino)propyl] guanidine hydrochloride (DOPG).

PEG coating of molecular weight 5000 was introduced to the interface of liposomes through the incorporation of varying amounts of PEGylated cholesterol in the liposomal membrane. This is a

convenient method of attaching PEG to the liposomal surface since the end of the polymer chain bearing the cholesterol moiety is effectively anchored inside the liposomal membrane. One of the highlights of this study is that molecular recognition of the complementary liposomes leads to the formation of either large aggregates, which precipitate, or to fused multicompartment aggregates, as measured by dynamic light scattering (DLS) and observed by phase-contrast microscopy (**Figure 7**). The results obtained by the two methods were in good agreement. The size increase during the interaction of PC:CHOL:DOPG with PC:CHOL:DHP liposomes in water is shown in **Figure 8**.

Fusion of complementary liposomes takes place under a non-leaking process involving lipid mixing as it was established by calcein entrapment and Resonance Energy Transfer experiments (Pantos et al, 2004). The thermodynamic parameters indicate the processes of aggregation and fusion. The interactions of non-PEGylated liposomes consistently involve exothermic processes of much higher enthalpy 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.

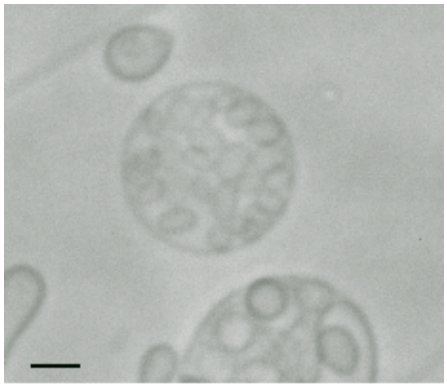


Figure 7. Multicompartment aggregates obtained following molecular recognition of complementary liposomes incorporating DHP and ODPG. The bar in the lower left corner indicates 5μm.

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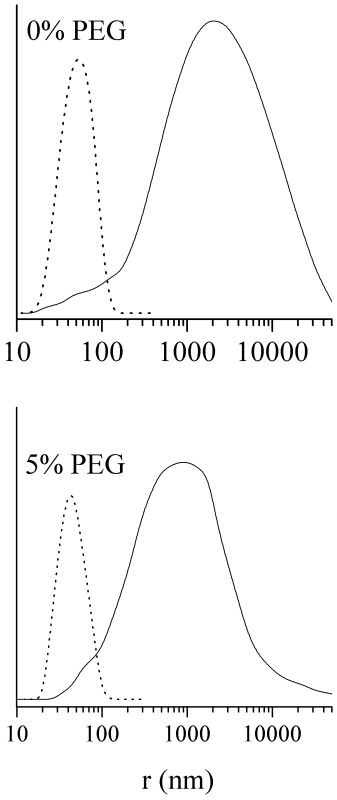

Figure 8. Particle size distribution of unilamellar PC:CHOL:DOPG liposomes (dotted line) and of the resulting aggregates following their interaction with unilamellar PC:CHOL:DHP liposomes (PEGylated or not) in water.

On the basis of the results obtained from liposome-liposome interactions, a model for the interaction of drug-loaded liposomes with cells was constructed. This was simulated by the interaction of drug-loaded unilamellar liposomes with multilamellar liposomes in which the latter play the role of cells (Paleos et al, 2001). In these experiments the same lipids, as in previous work (Pantos et al, 2004) were used. The interaction of unilamellar with multilamellar liposomes was investigated by optical microscopy, DLS, ζ-potential, high-precision differential scanning calorimetry, and ITC experiments (Pantos et al, 2005a). Multicompartment systems were observed by means of optical microscopy as shown in **Figure 9**, consistent, in size with that determined by DLS experiments, **Figure 10**. This further supports the hypothesis that molecular recognition induces multicompartment system formation. The aggregates observed are analogous to the ones obtained when unilamellar recognizable liposomes were allowed to interact as previously discussed.

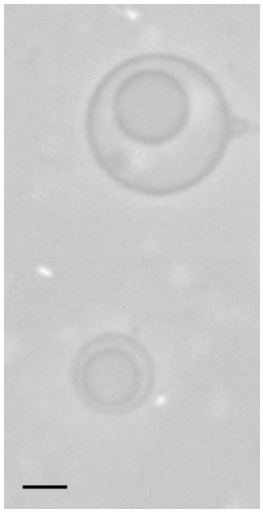

Figure 9. Phase contrast optical microscopy images of liposomal aggregates obtained following the mixing of multilamellar PC:CHOL:DHP liposomes with complementary unilamellar PC:CHOL:ODPG liposomes. The bar in the lower right corner indicates 5 μm.

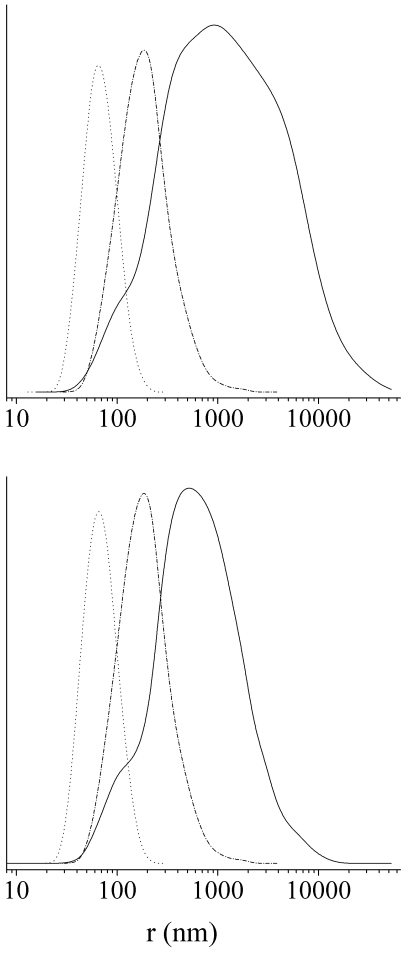

Figure 10. Particle size distribution of PEGylated (upper part) and non-PEGylated (lower part) unilamellar (dotted line) PC:CHOL:ODPG liposomes and of the resulting aggregates (solid line) following interaction with PC:CHOL:DHP multilamellar liposomes (dashed-dotted line) at a 2:1 DHP:ODPG molar ratio. Reproduced from Paleos and Tsiourvas, 2006 with kind permission from Wiley-VCH.

By a closer examination of multicompartment system formation, as an outcome of molecular recognition between liposomes, the following mechanism may be envisaged: Fusion between liposomes is facilitated by their recognizable functional groups affording liposomes of various sizes (interaction steps A and B in **Figure 11**), which are in general characterized as large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV). According to Lehn et al, (Marchi-Artzner et al, 2001) who employed a similar complementary pair of liposomes, adhesion leading to fusion does not take place between giant liposomes, while a selective LUV-GUV adhesion can take place leading to fusion (interaction step C in **Figure 11**). This last step can rationalize the formation of multicompartment systems, during which large liposomes are recognized, engulfed and internalised by giant liposomes, in a fashion analogous to endocytosis. Aggregates, analogous to the ones presented in this review, were obtained while a mechanism of membrane fusion was proposed elsewhere (Tanaka and Yamazaki, 2004) explaining the incorporation of smaller liposomes into the larger ones.

Coming to the issue of simulating the interaction of cells with liposomes loaded with drugs, unilamellar liposomes were loaded either with the hydrophilic drug doxorubicin (DXR) or with the hydrophobic drug tamoxiphen (TMX) (**Figure 12**) and allowed to interact with multilamellar liposomes (Pantos et al, 2005a). It should be noted that all the experiments were carried out in phosphate buffer saline (PBS) in order to approximate physiological conditions. The interaction of the complementary non-PEGylated unilamellar liposomes loaded with DXR or TMX with multilamellar liposomes, at 1:2 molar ratio with respect to PC, occurred spontaneously. Large aggregate precipitates were obtained as imaged by phase contrast optical microscopy. A few giant fused liposomes were also observed which, in some cases, encapsulated smaller aggregates. When PEGylated unilamellar liposomes were used, precipitation was reduced while the occurrence of fused species was increased.

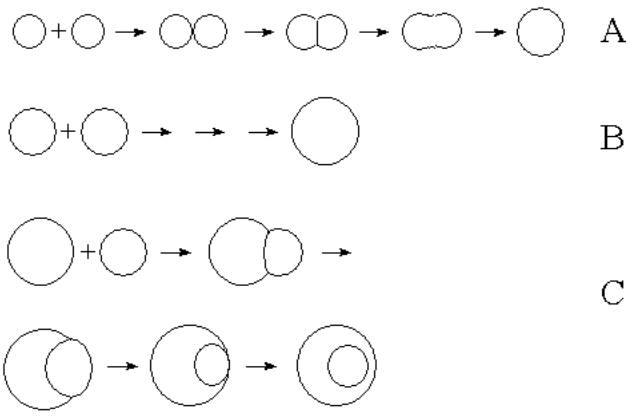

Figure 11. Schematic diagram of multicompartment aggregates formation steps by the interaction of complementary liposomes.

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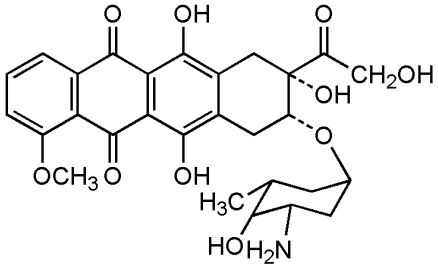
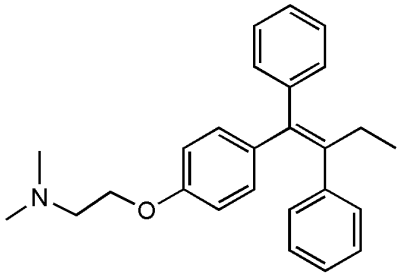
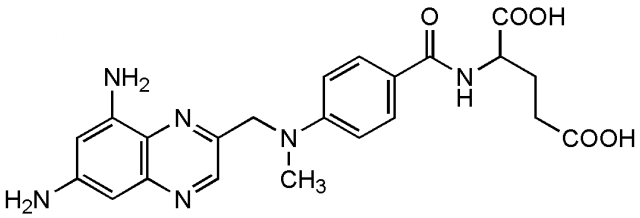


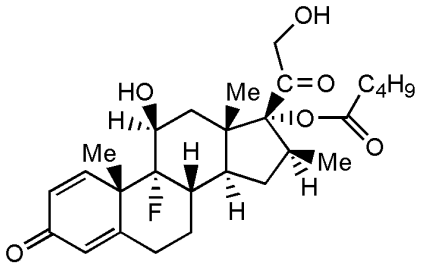
DXR



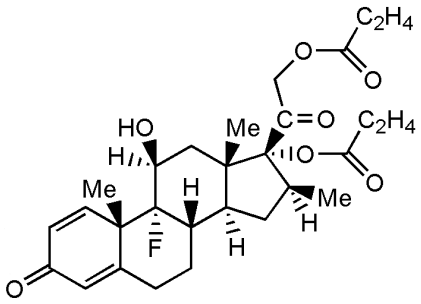
TMX



MTX



BV



BD

Figure 12. Chemical structure of the drugs doxorubicin, ADR, methotrexate, MTX, betamethasone valerate, BV, and betamethasone dipropionate, BD.

The results of the DXR transport from drug loaded unilamellar liposomes to the 'empty' complementary

multilamellar liposomes during their interaction are summarized in **Table 1**. The control experiments demonstrate a rather minor drug transport, not higher than 17%, in the case of simple, PEGylated or non-PEGylated, unilamellar liposomes. In contrast, when PEGylated or non-PEGylated unilamellar liposomes bearing the recognizable ODPG lipid were used, drug transfer increased by a factor of 4 and 4.5 respectively compared to the control experiments, i.e, liposomes without any recognizable moieties on their surface. This was attributed to the formation of aggregates or giant liposomes as a result of molecular recognition between the complementary moieties of the unilamellar and multilamellar liposomes. The observed variation between the PEGylated and non-PEGylated unilamellar liposomes can be attributed to the presence of polymer chains at the external liposomal surface hindering adhesion as also previously established in the case of unilamellar interacting liposomes (Pantos et al, 2002, 2004).

Drug transport experiments were also performed with the TMX loaded unilamellar liposomes, demonstrating analogous results as summarized in **Table 1**. In this case drug transport is even higher, by 85% and 90 %, when PEGylated and non-PEGylated unilamellar liposomes incorporating ODPG were used respectively. In contrast, control experiments clearly show that drug transfer for unilamellar liposomes non-incorporating ODPG is insignificant, i.e. less than 2%.

The observed differences in the transport efficacy of TMX and DXR, can be attributed to their different mode of incorporation into the unilamellar liposomes. Specifically, the hydrophilic DXR is encapsulated into the liposomal aqueous core while the hydrophobic TMX is incorporated into the liposomal bilayer. In control experiments DXR transfer is not negligible, in contrast to TMX. This is attributed to its higher water solubility and diffusion through the aqueous phase to the multilamellar liposomes. Nevertheless, during interaction of the complementary liposomes drug transfer of DXR into the multilamellar liposomes is less than that of TMX due to its release and solubilization in the aqueous phase. Conversely since TMX is solubilized in the bilayer, its transport does not involve leakage or diffusion through the aqueous medium, and therefore it is higher than DXR in the case of complementary liposomes and insignificant in the case of non-functionalized ones.

III. Interaction of functional dendritic polymers with complementary liposomes

Dendrimers (Bosman et al, 1999; Schlüter and Rabe, 2000; Fréchet and Tomalia, 2001; Newkome et al, 2001; Jiang and Aida, 2005; Tomalia, 2005) are nanoscale, highly branched and monodispersed macromolecules with symmetrical architecture. They consist of a central core, branching units and terminal functional groups. The core and the internal units determine the nanocavity environment and consequently the dendrimer solubilizing or encapsulating properties, whereas, the external groups influence their solubility and chemical behaviour. On the other hand, hyperbranched polymers (Inoue, 2000; Muscat and van Benthem, 2001; Voit, 2003), including the extensively studied hyperbranched polyether polyols or polyglycerols (Sunder et al, 1999a,b, 2000a,b; Haag, 2001; Frey and Haag, 2002; Siegers et al, 2004) are readily prepared but are non-symmetrical, highly branched and polydispersed. Their main structural feature, also common to dendrimers, is the formation of nanocavities able to encapsulate bioactive molecules, among others.

In this context, commercially available or custom-made dendrimeric or hyperbranched polymers have been functionalized (Vögtle et al, 2000) for their application as effective drug delivery systems (Liu and Fréchet, 1999; Sideratou et al, 2001; Stiriba et al, 2002; Beezer et al, 2003; Kolhe et al, 2003; Ambade et al, 2005; D' Emanuele and Attwood, 2005; Gillies and Fréchet, 2005; Lim and Simanek, 2005; Dhanikula and Hildgen, 2006; Tziveleka et al, 2006; Paleos et al, 2007) or gene vectors (Bielinska et al, 1999; Luo et al, 2002; Ohsaki et al, 2002; Dufès et al, 2005; Lee et al, 2005; Svenson and Tomalia, 2005; Liu and Reineke, 2006; Tziveleka et al, 2007). When more than one type of groups is introduced to the surface of dendritic polymers, these systems are characterized as multifunctional as shown in **Figure 1**. Each group serves a specific function when these multifunctional dendritic polymers are employed as drug delivery systems. Thus, specificity for certain cell types can be accomplished by attaching targeting ligands to the surface of dendritic polymers, while enhanced solubility, decreased toxicity, biocompatibity, stability and protection from degradation in the biological milieu can be achieved by the functionalization of the end groups of dendritic polymers, e.g. with poly(ethylene glycol) chains (PEG).

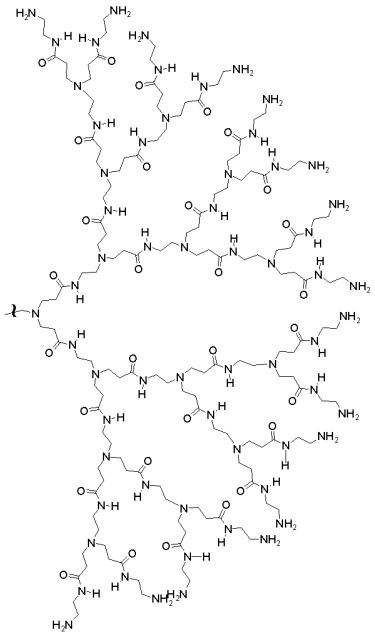
Table 1. Drug transport from the unilamellar to the multilamellar liposomes. Reproduced from Pantos et al, 2005a with kind permission from American Chemical Society.

Composition of unilamellar liposomes	DXR % transfer	TMX % transfer
PC-CHOL	$17.1_{\pm 2.9}$	<2
PC-CHOL-PEG	$12.5_{\pm 3.4}$	<1
PC-CHOL-ODPG	$70.5_{\pm 3.9}$	$94.9_{\pm 3.1}$
PC-CHOL-ODPG-PEG	$60.9_{\pm 2.3}$	$82.5_{\pm 3.9}$

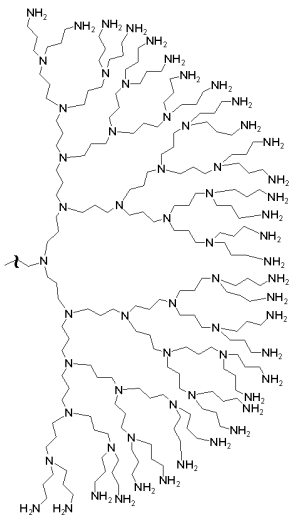
The function of PEG-chains is crucial for modifying the behaviour of drug themselves or their carriers (Noppl-Simson and Needham, 1996; Ishiwata et al, 1997; Liu et al, 1999, 2000; Veronese, 2001; Roberts et al, 2002; Pantos et al, 2004; Vandermeulen and Klok, 2004; Vonarbourg et al, 2006; Gajbhiye et al, 2007).

Targeting ligands attached to the dendritic surface are complementary to cell receptors (Cooper, 1997; Lodish et al, 2000) in order to induce binding of the dendritic carrier to the cell surface and efficient internalisation. This binding is further enhanced by the so-called polyvalent interactions (Mammen et al, 1998; Kitov and Bundle, 2003; Badjic et al, 2005) attributed to the close proximity of the recognizable ligands situated on the limited surface area of the dendritic molecules. Transport through the cell membrane can also be enhanced by the introduction of appropriate moieties to the surface of the dendritic polymers. Moreover, modification of the internal groups of dendrimers affects their solubilizing character, making therefore the encapsulation of a pleiad of drugs possible. In this connection, cationization of dendrimers, and particularly of their external groups, facilitates their application as gene transfer agents (Bielinska et al, 1999; Luo et al, 2002; Ohsaki et al, 2002) through the formation of DNA-Dendritic Polymer complexes.

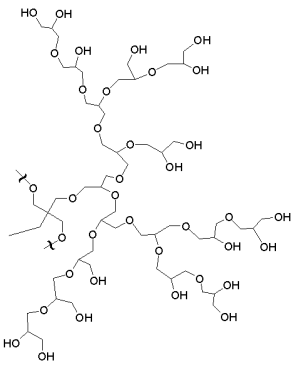
As expected monofunctional dendritic drug carriers cannot compete with their multifunctional counterparts. Thus selected monofunctional dendrimeric polymers such as poly(amidoamine), PAMAM, diaminobutane poly(propylene imine), DAB, or the hyperbranched polyglycerol, PG (**Figure 13**) have been step-wise multi-functionalized. In order to illustrate dendrimeric encapsulation, a few typical examples are given below: PAMAM functionalized with monomethyl ether poly(ethylene glycol) (M-PEG), having an average molecular weight of 550 or 2000, was employed for encapsulating the anticancer drugs Adriamycin, ADR (Doxorubicin hydrochloride), or Methotrexate, MTX, (**Figure 12**) (Kojima et al, 2000), while PEGylated DAB dendrimers were used to encapsulate betamethasone valerate (BV) and betamethasone dipropionate (BD) (**Figure 12**) (Sideratou et al, 2001). It should be noted that BV loading capacity was found to be of the order of 11 wt% in multi-functional dendrimers, i.e. in PEGylated and Guanidinylated derivative (Paleos et al, 2004), which is almost double the loading capacity of the simply PEGylated dendrimer (6 wt%) (Sideratou et al, 2001) and more than 5fold the loading capacity of the parent dendrimeric solution (1.7 wt%). The encapsulation of a diversity of conventional drugs in functional dendritic polymers and the formation of Dendritic-DNA complexes constitute a prospective and very promising new field in drug delivery. The progress in this field has been recently reviewed (Sideratou et al, 2006). In the present review however, the interaction of dendritic polymers with their complementary liposomes is proposed as a drug delivery model mimicking the interaction of functional dendritic polymers with cells. As previously discussed, multilamellar liposomes interacting with multifunctional dendritic polymers were used instead of cells.



PAMAM



DAB



PG

Figure 13. Chemical structure of dendritic polymers.

Guanidinylated diaminobutane poly(propylene imine) dendrimers of the fourth and fifth generation acted as a "glue" causing the aggregation of phosphatidylcholine-cholesterol liposomes incorporating dihexadecylphosphate as the recognizable lipid; these aggregates were readily redispersed by the addition of an excess of a phosphate buffer (Sideratou et al, 2002b). These initial results on molecular recognition of DHP bearing liposomes with guanidinylated dendrimers prompted the development of an elaborated multifunctional dendrimer (Paleos et al, 2004) based on a fifth generation DAB (**Figure 14**). The design of this dendrimeric carrier was intended to simultaneously address issues such as stability in the biological milieu, targeting and transport through cell membranes. For this purpose, in addition to protective poly(ethylene glycol) chains, guanidinium moieties were also introduced to the dendrimer surface, acting both as targeting and transport ligands. In this manner it was possible to achieve, the key features of an efficient drug delivery system: a. Protection of the carrier through coverage of the dendrimeric surface with poly(ethylene glycol) chains, b. Recognition ability towards complementary moieties as surface guanidinium groups secure the facile interaction with acidic receptors, including the biologically significant carboxylate and phosphate groups. The combination of electrostatic forces and hydrogen bonding that occur make this interaction thermodynamically favorable (Hirst et al, 1992), c. Potential of encapsulation of various drugs in the dendrimer nanocavities and subsequent release from them, possibly in a controlled fashion which can be tuned by environmental changes (Sideratou et al, 2001), d. Complexation with DNA for gene therapy applications, e. Capability of polyvalency interactions, associated with enhanced binding, due to the high density of recognizable moieties on the confined surface area of the dendrimer, which can significantly enhance translocation through a bilayer membrane. f. An anticipated decrease in toxicity due to the modification of the toxic amino groups (Malik et al, 2000).

In order to investigate the translocation ability of DAB dendrimers across liposomal bilayers, a series of derivatives bearing varying numbers of guanidinium groups on their surfaces were prepared. At low guanidinium/phosphate molar ratios, i.e. when weakly guanidinylated dendrimeric derivatives were employed, the aggregate formation was imaged with AFM microscopy while liposomal fusion occured to a certain extent at high guanidinium/phosphate molar ratios or when highly guanidinylated dendrimeric derivatives were employed (Tsogas et al, 2006). Furthermore, optimal translocation of these dendrimeric derivatives to the liposomal core was shown through fluorescence for low to medium guanidinylation and at low guanidinium/phosphate molar ratios; translocation was further enhanced when the lipid bilayer was in the fluid liquid crystalline phase. In conclusion, an optimum balance is required between the recognition effectiveness as expressed by the number of guanidinium groups interacting with the phosphate groups and the degree of hydrophilicity of the guanidinylated dendrimers for optimum transport of the latter to the liposomal core (Tsogas et al, 2006).

Having discussed the processes involved in the interaction of liposomes with complementary dendrimers their interaction with cells needs to be modelled next. Multilamellar liposomes consisting of PC:CHOL:DHP (19:9.5:1) and dispersed in aqueous or phosphate buffer solutions (Pantos et al, 2005b) were employed to simulate cells interacting with multifunctional dendrimers. Specifically, poly(propylene imine) dendrimers of the fourth generation were functionalized with 6 (DAB-G6) or 12 (DAB-G12) guanidinium groups as targeting ligands, while, the remaining toxic, external primary amino groups of the dendrimers reacted with propylene oxide affording the corresponding hydroxylated derivatives (**Figure 15**). The fully hydroxylated dendrimer DAB-G0 not containing any guanidinium groups was used as a reference compound. All dendrimers were loaded with corticosteroid drugs, i.e. betamethasone dipropionate and betamethasone valerate in order to evaluate their drug transfer efficiency to liposomes.

Microscopy, ζ-potential, and Dynamic Light Scattering (DLS) revealed liposome-dendrimer molecular recognition leading to the formation of large aggregates at dendrimer/DHP molar ratios higher than 1:30. Calcein liposomal entrapment experiments demonstrate limited leakage (less than 13%) following liposome interaction with modified dendrimers at 1:25 dendrimer/DHP molar ratio. This indicates that liposomal membranes remain almost intact during their molecular recognition with these dendrimers. Isothermal Titration Calorimetry (ITC) indicates that the enthalpy of the interaction is dependent on the number of the guanidinium groups present on the dendrimeric surface. Furthermore, the process is reversible and redispersion of the aggregates occurs by adding a high concentration of phosphate anions.

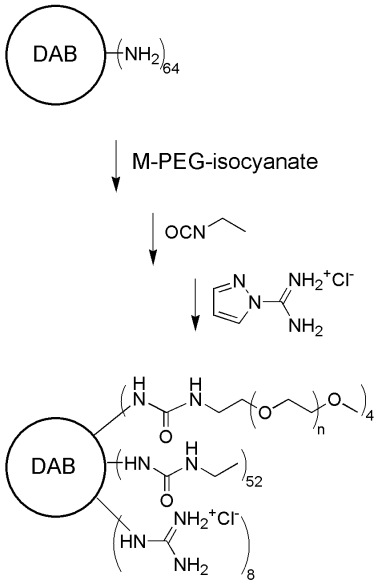


Figure 14. Reaction scheme for the synthesis of a multifunctional dendrimeric derivative.

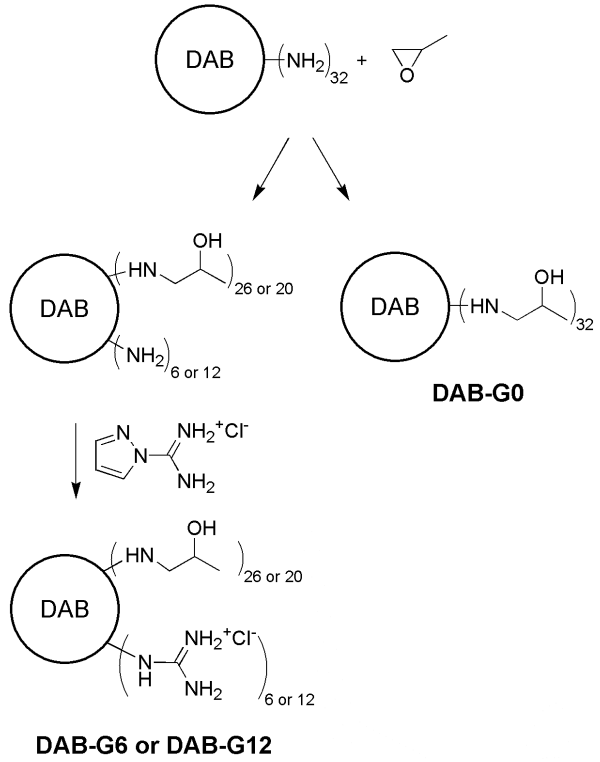


Figure 15. Multi-functionalization reaction scheme of fourth generation poly(propylene imine) dendrimer.

The interaction between drug-loaded dendrimers and multilamellar liposomes results in drug transport from the dendrimeric derivatives to the 'void' multilamellar liposomes as summarized in **Table 2**. These experiments showed that about 25% of BD or BV was present in the precipitated aggregates when DAB-G0 was used. When the guanidinylated dendrimers DAB-G6 and DAB-G12 were used, the amount of drugs in the precipitate profoundly increases to about 60% and 80%, respectively.

These significant differences observed in the transport of drugs between guanidinylated and non-guanidinylated dendrimers can be attributed to the functionalization of the dendrimeric molecules. The presence of guanidinium groups at the external surface of the dendrimers results in effective adhesion to the multilamellar liposomes as the ITC and DLS experiments demonstrated. As expected, when the interaction is performed in 10mM phosphate buffer the amount of drug present in the aggregates slightly decreases. This decrease can be explained by the competitive interaction of the phosphate groups in the bulk water phase with the dendrimeric guanidinium groups, leading to less effective adhesion to the multilamellar liposomes.

Upon addition of concentrated phosphate buffer followed by the redispersion of the aggregates in the medium and the separation of the no-longer interacting dendrimers, there is still some drug present in the detached multilamellar liposomes. Measurements of BD or BV found in multilamellar liposomes indicate that, in all cases, ca. 50% (**Table 2**) of the amount of drugs found in the aggregates before redispersion is still present, suggesting that both drugs are incorporated in the liposomal lipid bilayer, since their solubility in water is extremely low. Drug transport is highly enhanced by the use of guanidinylated dendrimers since drug transfer of 40-45% was obtained in the case of DAB-G12 while the corresponding transfer for the non-guanidinylated derivative was merely 12-15%.

IV. Concluding remarks

The preparation and physicochemical characterization of multifunctional liposomal and dendritic nanoparticles intended for application as drug delivery systems was presented. The multifunctionality together with the multivalency capability of both categories of nanoparticles gives these systems increased potential as effective drug delivery systems. In view of these features and employing multilamellar liposomes, to simulate cells, it was possible to model the processes occurring during the interaction of liposomal and dendritic nanoparticles with cells. A key feature of this model is molecular recognition of the nanoparticles involved leading to adhesion, which is expected to enhance drug transport.

Acknowledgements

The work was partially supported by Dendrigen SA, Athens, Greece.

Table 2. Drug transfer (%) from dendrimers to multilamellar liposomes in a) aggregates obtained after their interaction in water or in 10 mM phosphate buffer (pH 7.4) and b) multilamellar liposomes obtained following redispersion of the aggregates. Reproduced from Pantos et al, 2005b with kind permission from American Chemical Society.

Drug	Dendrimer	Drug transfer (%) in aggregates		Drug transfer (%) after redispersion	
		Water	Phosphate Buffer	Water	Phosphate Buffer
BD	DAB-G0	$24.4_{\pm 2.4}$	$19.8_{\pm 1.2}$	$15.8_{\pm 0.9}$	$12.1_{\pm 1.1}$
	DAB-G6	$62.5_{\pm 1.9}$	$48.5_{\pm 1.6}$	$28.1_{\pm 1.7}$	$24.5_{\pm 1.3}$
	DAB-G12	$84.5_{\pm 2.1}$	$68.4_{\pm 1.5}$	$45.1_{\pm 1.8}$	$40.0_{\pm 1.4}$
BV	DAB-G0	$32.9_{\pm 2.0}$	$27.1_{\pm 1.0}$	$15.9_{\pm 1.2}$	$14.1_{\pm 0.9}$
	DAB-G6	$59.0_{\pm 1.5}$	$39.5_{\pm 2.1}$	$29.0_{\pm 1.0}$	$26.1_{\pm 1.5}$
	DAB-G12	$78.1_{\pm 2.3}$	$57.5_{\pm 2.0}$	$42.0_{\pm 1.5}$	$38.2_{\pm 1.2}$

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