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Phase Transformation of a Liposomal Dispersion into a Micellar Solution Induced by Drug-Loading

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Purpose. Loading a liposomal dispersion with drug may cause a phase transformation into a micellar solution. The present contribution presents a detailed physicochemical characterization and an overall model which describes transformation due to the properties of any drug.
Methods. Characterization of liposomal dispersions was obtained by photon correlation spectroscopy (PCS) and small angle X-ray scattering (SAXS). Microstructure of colloidal solutions was analysed by ^{31}P-NMR and SAXS.
Results. At weight ratios of phospholipid to drug from 16:1 to 2:1, liposomal dispersions of milky-white appearance and a mean particle size of about 200 nm were obtained. From a ratio of phospholipid to drug of 1:1 downwards, the systems became nearly transparent. The particle size decreased to a value below 25 nm. SAXS also revealed the change of the colloids. Down to a ratio of phospholipid to drug of 2:1 the systems were described as bilayer-structured. At and below the ratio of 1:1, a mixed micelle was indicated. In the ^{31}P-NMR spectra, the transformation is emphasized by both appearance and disappearance of signals. A model based on the theory of self-assembly is presented which explains the phase transformation due to drug amphiphilicity.
Conclusions. We predict that the model presented will hold in general only due to the amphiphilic properties of the drug.

KEY WORDS: liposomes; colloidal drug carriers; small angle X-ray scattering (SAXS); self-assembly.

INTRODUCTION

The use of colloidal drug carrier systems may reduce side effects of drugs, because changes in structure and/or physicochemical properties lead to a different in vivo behaviour (targeting specific sites) (1). Furthermore, the use of colloidal drug carrier systems allows the control of the in vivo release pattern.

Liposomes are a promising colloidal drug carrier system because they consist of phospholipid and are thus nontoxic and biodegradable (1). Furthermore, their good solubilization power and capacity for drugs and the relative ease in preparation make them a very interesting drug carrier system. Another advantage is their wide range of applicability from i.v. administration to topic administration. Due to the fact that colloidal carriers are very rarely administered of their own but bear a drug load instead, it is important to know whether the physicochemical properties and the structure of the liposomal dispersions are changed by drug loading. The magnitude and nature of these changes have to be analysed. Diclofenac sodium, which is a widely marketed nonsteroidal antiinflammatory drug, was chosen as a model drug because of its known amphiphilic and mesogenic properties (2), the latter of which make it a promising candidate for interactions with liposomes. A model which is able to predict change in the physicochemical properties and structure of liposomal dispersions due to the chemical properties of the incorporated drug will be a useful tool for the development of new colloidal drug carrier systems.

MATERIALS AND METHODS

Materials

Liposomal Dispersions

Appropriate amounts of Natipide ^{R}II (Rhône Poulenc Rorer, Cologne, Germany), drug and water were weighed into vessels. All components were dispersed by stirring with a magnetic stirrer (100 rpm) for 2 hours at room temperature. All liposomal dispersions had a constant phospholipid content of 6% (w/w). The following drug-loaded samples were prepared (all w/w): 16:1, 8:1, 4:1, 2:1, 1:1, 1:2 (ratio of phospholipid to drug) and a non-loaded dispersion.

Natipide^{R}II

Natipide^{R}II is a semi solid, transparent gel of close packed multilamellar vesicles (3). Natipide^{R}II contains about 20% phospholipid, about 18% ethanol and up to 100% demineralized water. The phospholipid is a soyabean phospholipid Phospholipon^{R}80 (Rhône Poulenc Rorer, Cologne, Germany) with a high content of unsaturated fatty acids. The main component is linoleic acid.

Drug

Diclofenac sodium as well as, for comparative purposes only, diclofenac acid and diclofenac diethylamine were kindly provided by Ciba-Geigy (Basel, Switzerland). Since the pKa of the free acid is 3.8 (4), the sodium salt forms aqueous solutions of pH 6.5 (5). The latter also holds for diethylamine, of which the solubility in water at 20°C is highest.

Reference for Mixed Micellar System

Phospholipon G 90 (Rhône Poulenc Rorer, Cologne, Germany), water, and drug were dispersed by ultrasonication (Soniprep 150, MSE, Scientific Instruments, Crawley, United Kingdom) under controlled conditions at $T = 20°C$ and sonication-interval 60s/on and 60s/off for 60 min. All samples were in phase equilibria, thermodynamically stable for at least 1 year, stored in an air-conditioned room ($T = 20°C \pm 1°C$, 70%±2% rel. humidity) and preserved with thiomersal 2.10^{-5}% (w/w).

METHODS

Photon Correlation Spectroscopy (PCS)

The studies were performed with a Zetasizer 3 (Malvern Instruments, Malvern, United Kingdom). The sample holder AZ10 (scattering angle of 90°) and the standard HeNe-laser ($\lambda = 632.8nm$) with 5mW power were used. Data collection and

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analysis were done by Malvern K 7032 CN correlator and Malvern software (release 1.1). The given values are mean z-average values of 5 runs (each run consists of 10 single measurements). All measurements were performed at $T = 20°C$. All samples were measured without prior dilution.

Small Angle X-ray Scattering (SAXS)

The SAXS measurements were carried out with a Kratky-Kamera (6) (Anton Paar, Graz, Austria). The radiation source was a Kratky broad focus tube (PW 2253/11) with Cu-anode ($\lambda = 1.54$ Å) (Philips, Hamburg, Germany) which was mounted on a PW1830 X-ray generator (Philips, Hamburg, Germany). The scattered radiation was detected with a position sensitive detector (PSD) (OED-50M) and the signals were amplified and analysed by a multichannel analyser unit (all MBraun, Munich, Germany). Data collection was done by the MCD software (release 1.90) (MBraun, Munich, Germany).

The samples were measured in a capillary holder K-PR with peltier temperature control (Anton Paar, Graz, Austria). The measurements were performed in a vacuum ($p = 10^{-2}$ Torr) at $T = 20°C \pm 0.1°C$ and exposition time was $t = 5000$s.

For data analysis, the ITP-81/DECON program package was used which was developed by O.Glatter (7,8). The calculations were carried out on the mainframe IBM3090 600J of the Technische Universität Braunschweig and on a Hewlett Packard Apollo 713/33.

The application of SAXS to colloidal drug carrier systems is described elsewhere (9).

^{31}P-NMR

Spectra were recorded on a Bruker AC200 spectrometer. H_3PO_4 (85% (w/w) was used as an external reference and C_6D_6 as an internal standard. The measurements were performed at 81.0 MHz and $T = 20°C \pm 1°C$.

RESULTS

Even on first sight, the change in the properties of the liposomal dispersions at various ratios of phospholipid to drug was recognized. Ratios of phospholipid to drug of 2:1 and above resulted in a milky-white appearance, and from a ratio of phospholipid to drug of 1:1 and below, the systems were nearly transparent. Analysis of the particle size (z-average value) revealed that the particle size decreases with increasing drug content until a ratio of phospholipid to drug of 2:1 was reached (Table 1). At a ratio of phospholipid to drug of 1:1 and below, the z-average value was drastically lowered to less than 25 nm.

To get information in detail where the drug molecule was situated within the liposomes, ^{31}P-NMR-measurements were performed. Due to the amphiphilicity of the drug, it should have been incorporated next into the hydrophilic headgroup of the phosphatidylcholine molecule. The basic idea was to use the ^{31}P-NMR spectra of the non-loaded liposomal dispersion as a 'reference' and to check how the ^{31}P-NMR spectra were changed by introducing certain amounts of the amphiphilic drug into the dispersions (Table 2). The liposomal dispersion showed only one signal in the spectra at 0.3 ppm, whereas the ^{31}P-NMR spectra of liposomal dispersions with ratios of phospholipid to drug from 16:1 to 2:1 exhibited three signals, clearly indicating the change in the surrounding area of the phosphorus atom due to the incorporation of drug next to the hydrophilic headgroup of the phosphatidylcholine molecule. The signal of the non-loaded dispersion was still present in the ternary systems although slightly shifted to a higher field by 0.02 ppm, while the two additional ones were strongly highfield shifted in comparison with the 'reference'. The liposomal dispersions with a ratio of phospholipid to drug of 1:1 and 1:2 respectively showed completely different ^{31}P-NMR spectra (Table 2). The 'reference' signal at about 0.3 ppm vanished, but the two highfield shifted signals still remained.

Table 1. Particle Size (z-Average Value) and Bilayer Thickness (Obtained by Analysing the SAXS-Measurements Due to the Flat Symmetry Assumption) of the Liposomes

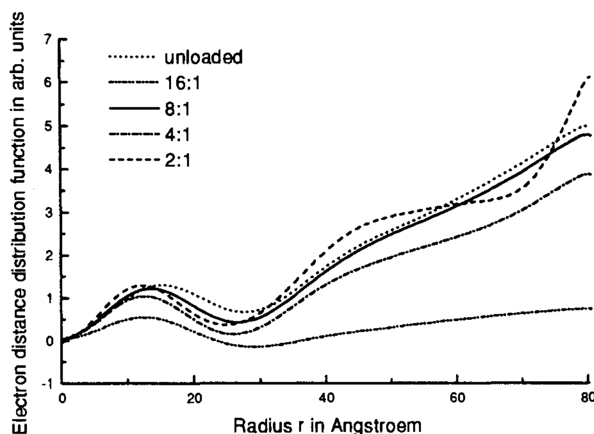
Ratio of phospholipid to drug	Particle size (z-average) in nm	Bilayer thickness in Å
non-loaded	211.0 ± 1.1	64
16:1	220.6 ± 5.5	55
8:1	194.0 ± 4.5	53.5
4:1	172.3 ± 0.6	51
2:1	155.8 ± 0.9	49.5
1:1	12.9 ± 1.2	–
1:2	20.5 ± 3.3	–

Prior to data analysis of the SAXS measurements, spherical (three-dimensional) symmetry for the scattering object was postulated since liposomes themselves are spherical. The resulting $p(r)$ functions for the liposomal dispersions with ratios of phospholipid to drug of 16:1, 8:1, 4:1, and 2:1, respectively, and for the non-loaded dispersions are shown in Fig. 1A. All $p(r)$ functions show oscillations. These oscillations are a strong hint of electron density inhomogenities within the scattering object (10) which in this particular case belong to the hydrophilic headgroup of the phosphatidylcholine molecule (higher electron density than water) and the lipophilic chains (lower electron density than water). Furthermore, the $p(r)$ function provides information about the dimensions of the scattering object, i.e., the $p(r)$ function has to become zero when the maximum distance within a particle is reached. For all curves, however, the $p(r)$ function vanishes to infinity. Therefore, it is not possible to determine the particle-size (diameter) assuming spherical symmetry for the scattering object in all liposomal dispersions with a ratio of phospholipid to drug from 16:1 to 2:1 and in the non-loaded dispersion. The zero transition at about 25 Å

Table 2. ^{31}P-NMR Signals

Ratio of phospholipid to drug	Signal no. 1	Signal no. 2	Signal no. 3
non-loaded 0	0,30	-	-
16:1	0,31	0,05	−0,40
8:1	0,29	0,00	−0,39
4:1	0,29	0,00	−0,39
2:1	0,28	0,00	−0,37
1:1	—	0,07	−0,35
1:2	—	0,01	−0,41

A



B

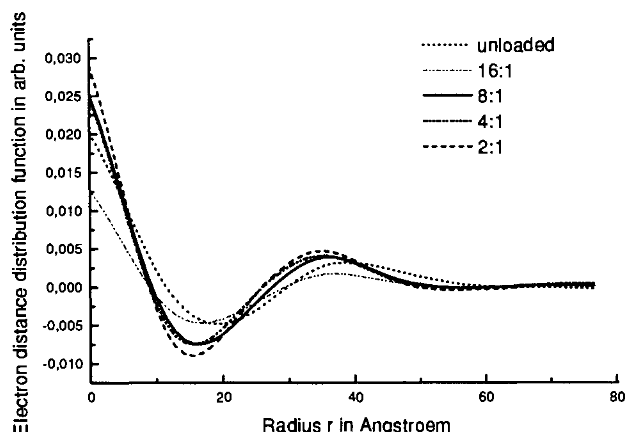
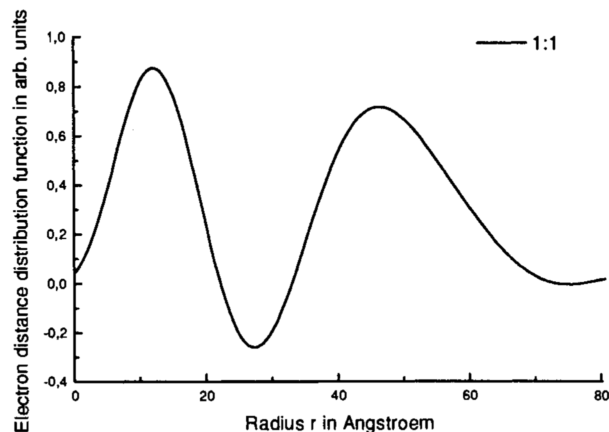


Fig. 1. (**A**) electron distance distribution functions $p(r)$ assuming spherical symmetry of the liposomal dispersions with a ratio of phospholipid to drug from 16:1 to 2:1 and the non-loaded system and (**B**) electron distance distribution functions $p(r)$ assuming flat symmetry of the liposomal dispersions with a ratio of phospholipid to drug from 16:1 to 2:1 and the non-loaded system.

A



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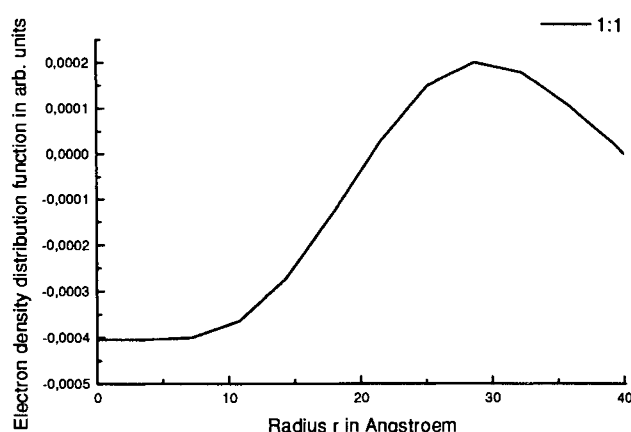

Fig. 2. (**A**) electron distance distribution functions $p(r)$ assuming spherical symmetry of the dispersion with a ratio of phospholipid to drug of 1:1 and (**B**) electron density distribution function $p(r)$ of the dispersion with a ratio of phospholipid to drug of 1:1.

cannot be attributed to the particle dimensions. It may be due to the electron density inhomogenities within the scattering object (see above).

Therefore, in a second step of data analysis a flat symmetry (one-dimensional symmetry) was postulated for the scattering objects. Thus, each bilayer within each liposome should be considered as a single scattering object (11). The postulation of flat symmetry finally provides data which represent the thickness of each layer because the $p(r)$ function 'looks' perpendicular to the bilayer surface and has to be zero when the distance vector r reaches the value of the layer thickness. In Fig. 1B, the resulting $p(r)$ functions for flat symmetry are shown. The oscillations in the curves are again due to the electron density inhomogenities within each bilayer (polar headgroup, lipophilic chains, lipophilic chains, polar headgroup). Thus, the maximum particle dimension, i.e. the bilayer thickness, is not given by the first zero transition at about 10 Å but in the region where the $p(r)$ functions converge to the x-axis at about 50–65 Å (depends on the sample, see Table 1).

The bilayer thickness decreases with increasing drug content. Furthermore, a shift of the first minimum at about 15 Å to lower values of the distance vector r with increasing drug content is recognized. Completely different results were obtained for the ternary systems with a ratio of phospholipid to drug of 1:1 and 1:2. Assuming a spherical model (three-dimensional symmetry) for the data analysis led to reasonable results, i.e. the dispersions were described by a spherical scattering object in contrast to the dispersions with ratios of phospholipid to drug from 16:1 to 2:1. As an example the resulting $p(r)$ function of a system with a ratio of phospholipid to drug of 1:1 is shown in Fig. 2A. There are two maxima at about 12 Å and 48 Å and a minimum at about 28 Å (zero transitions at about 21 Å and 32 Å). The $p(r)$ function tends to be zero (converge to the x-axis) at a value of about 72 Å. The latter

represents the maximum extent, i.e. the diameter, of the spherical scattering object. To get more information about the structure of this spherical scattering object the deconvolution of the $p(r)$ function was performed. The electron density distribution function $\rho(r)$ versus the distance vector is presented in Fig. 2B. The values at the y-axis are relative values, i.e., a value of $\rho(r) = 0$ refers to the electron density of the solvent (water). Hence, a negative value $\rho(r)$ refers to a region within the scattering object with an electron density lower than that of water, whereas a positive value of the electron density distribution function $\rho(r)$ corresponds to a region with a higher electron density than that of water. The $\rho(r)$ function results in an object of about 36 Å consisting of two parts with different signs of the electron density. Each part has an extent of 18 Å.

DISCUSSION

The results of the particle size measurements clearly showed the change in physicochemical properties by an incorporation of an amphiphilic drug into liposomal dispersions. A decrease in particle size with increasing drug content up to a certain ratio of phospholipid to drug was shown. A stepwise reduction of the particle size is probable, however not yet proven. From a ratio of phospholipid to drug of 1:1 downwards, particle sizes of less than about 25 nm were obtained. Bilayer-structured colloids cannot be that small because they would be thermodynamically not stable. So the scattering objects within the dispersions with a ratio of phospholipid to drug of 1:1 and 1:2 could not have been liposomes but mixed micellar systems. It should be pointed out that the decrease in particle size (Table 1) cannot be explained by the decrease of the bilayer thickness within each liposome (Table 1) (assuming 6–8 layers within each liposome (3)).

The ^{31}P-NMR results also confirmed the change in the colloidal arrangement. The non-loaded liposomal dispersion—as a reference—showed one sharp signal at 0.3 ppm. From the change of the headgroup signal of the phosphorus atom within the bilayer after loading with drug, it is concluded that the drug is indeed incorporated into the bilayer. A slight highfield-shift should occur according to the shielding of the ^{31}P core by the incorporation of the amphiphilic drug. At ratios of phospholipid to drug of 16:1 to 2:1, two additional highfield-shifted signals arose which were related to two 'new' structures. The signal at about 0.0 ppm might be related to size-reduced, drug-loaded liposomes, the one at about −0.4 ppm might be related to a mixed micellar system. At a ratio of 1:1 and below the reference signal at 0.3 ppm vanished whereas the signals at −0.4 ppm and 0.0 ppm remained. This was in accordance with the PCS-data which revealed a drastic decrease in particle size at this ratio. The comparison of the ^{31}P-NMR spectra of the dispersions with phospholipid to drug ratios of 1:1 and below with the spectrum of the mixed micellar reference system (Table 2) showed that the signal at −0.4 ppm had to be related to a mixed micellar system. However the question arises where the signal at about 0.0 ppm in the spectrum of the mixed micellar system came from. As an explanation, a noncomplete transformation of the liposomal dispersions may be considered. In this context, a few size-reduced liposomes may still be left over in the mixed micellar system. Their number has to be very low because they could not be detected by photon correlation spectroscopy. The mixed micellar reference system, however, did not reveal the signal at 0.0 ppm because it had never been in a liposomal state and therefore no transformation had occurred. A second question has to be asked as to why the signal of the mixed micelles occurs in the spectra with ratios of phospholipid to drug from 16:1 to 2:1. This may be due to the fact that at least low amounts of the drug already form a few mixed micelles. These micelles should not be recognized by PCS because their number is too low (12).

By means of SAXS, the structure of each colloid in the dispersion was analysed. For the liposomal dispersions at ratios of phospholipid to drug from 16:1 to 2:1, any particle sizes could not be detected by assuming spherical symmetry which would have been expected for liposomes. The flat symmetry approach, however, fitted the data best because the maximum particle size measured did not correspond to the diameter of the whole particle but to the thickness of each bilayer. The oscillations of the $p(r)$ functions (Fig. 1) agree well with the electron density profile perpendicular to the layer surface (polar headgroup, lipophilic chains, lipophilic chains, polar headgroup) (13). As far as the incorporation of the amphiphilic drug into the bilayer is concerned, the decrease in bilayer thickness with increasing drug content is due to interactions of the lipophilic regions of both the phospholipids and the drug molecules within the bilayer. Along with these interactions goes an increase in hydrocarbon chain tilt between the lipophilic regions. Similar results were obtained for the incorporation of diclofenac sodium into lyotropic lamellar liquid crystals (L_α-phase) of phosphatidylcholine (2). The repeat distance of the lamellar liquid crystal was reduced with increasing drug content.

The formation of a new associate at a critical ratio of phospholipid to drug of 1:1 was confirmed by the $p(r)$ function (Fig. 2A). The shape of the curve is typical for an aqueous micelle (two sharp maxima and one well defined minimum). The oscillations are again due to the electron density inhomogenities within the micelle. The electron density distribution function $\rho(r)$ confirms these results (Fig. 2B). It reveals a part with lower and with higher electron density in comparison with the electron density of the solvent. A model which explains the phase transformation of the liposomal dispersion into a mixed micellar solution is presented as follows.

In 1976 Israelachvili *et al.* [14], it pointed out the important role of geometric constraints in self assembly of micelles and bilayer vesicles composed of amphiphiles. A dimensionless 'packing parameter' $v/(a_o \cdot l_c)$ was defined where a_o is the surface area of the amphiphile, v the hydrocarbon chain volume, and l_c the critical chain length, which is less than the fully extended length of the chains, because the chains are above their chain melting temperature and hence in the liquid crystalline state (15). This 'packing parameter' determines whether spherical micelles at $v/(a_o \cdot l_c) < 1/3$, rod-like micelles at $1/3 < v/(a_o \cdot l_c) < 1/2$ or bilayers at $1/2 < v/(a_o \cdot l_c) \leq 1$ are formed. As a consequence of these geometric constraints, phospholipids do not form aqueous micelles because of the large 'packing parameter' according to the two chains (16). In Figs. 3A and 3B, a schematic representation of a phospholipid molecule (within a bilayer) together with its 'packing parameter' is shown. The incorporation of drug, especially of an amphiphilic drug, into the bilayer leads to a change of the 'packing parameter'. A modified packing parameter of two molecules, i.e. the

phospholipid and the drug, has to be considered. So the 'packing parameter' of the phospholipid alone of about 1 has to be substituted by a modified packing parameter (Fig. 3C). The value of the modified packing parameter decreases below 1/3 because the SAXS-measurements best agree with spherical symmetry. Hence the bilayer structure is no longer favourable. Spherical micelles arise consisting of both the amphiphilic drug and the phospholipid molecule. The introduction of a modified packing parameter should hold in general because just the amphiphilicity of the drug is considered. To test this model, a lipophilic drug, i.e. diclofenac in its acid form, was incorporated into the liposomal dispersions.

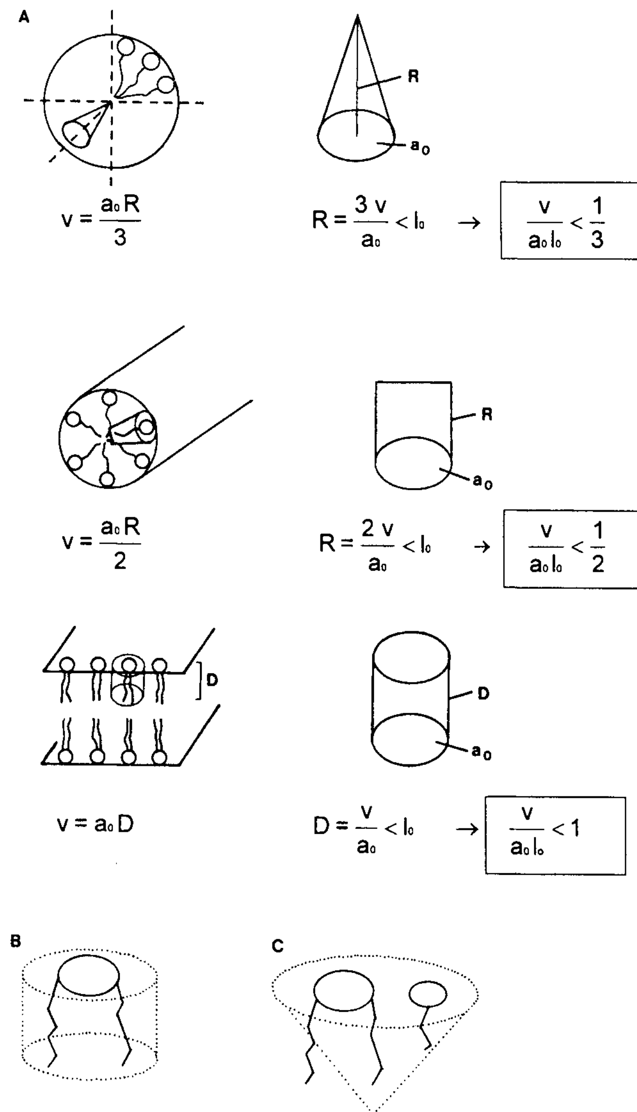

Fig. 3. (**A**) introduction of the 'packing parameter' [16], (**B**) 'packing parameter' for the phospholipid molecule within the non-loaded liposomal dispersion and (**C**) modified packing parameter for the phospholipid molecule plus amphiphilic drug molecule.

Due to the lipophilicity of the drug, it should be incorporated into the lipophilic part of the bilayer. The 'packing parameter' should not be changed much and the system should stay the same. Indeed, no phase transformation was observed. The incorporation of another amphiphilic drug, i.e. diclofenac diethylamine, led again to a phase transformation which was similar to the one observed for the incorporation of the amphiphilic diclofenac sodium.

CONCLUSIONS

Loading a colloidal carrier with an amphiphilic drug leads to a strong change in physicochemical properties. At a critical ratio of phospholipid to drug of 1:1, a phase transformation from a liposomal dispersion into a micellar solution occurs. The transformation can be explained by a model based on the theory of self assembly considering only the amphiphilic character of the drug. This model is predicted to hold in general which will be proven by further studies.

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