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Biophysical Chemistry 113 (2005) 183–192

Biophysical Chemistry

www.elsevier.com/locate/bpc

Development and initial evaluation of PEG-stabilized bilayer disks as novel model membranes

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Received 16 July 2004; received in revised form 13 September 2004; accepted 13 September 2004
Available online 29 September 2004

Abstract

We show in this study that stable dispersions dominated by flat bilayer disks may be prepared from a carefully optimized mixture of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-5000] [PEG-DSPE(5000)]. By varying the content of the latter component, the average diameter of the disks can be changed in the interval from about 15 to 60 nm. The disks show excellent long-term stability, and their size and structure remain unaltered in the temperature range between 25 and 37 °C. The utility of the disks as artificial model membranes was confirmed and compared to uni- and multilamellar liposomes in a series of drug partition studies. Data obtained by isothermal titration calorimetry and drug partition chromatography (also referred to as immobilized liposome chromatography) indicate that the bilayer disks may serve as an attractive and sometimes superior alternative to liposomes in studies aiming at the investigation of drug–membrane interactions. The disks may, in addition, hold great potential for structure/function studies of membrane-bound proteins. Furthermore, we suggest that the sterically stabilized bilayer disks may prove interesting as carriers for in vivo delivery of protein/peptide, as well as conventional amphiphilic and/or hydrophobic, drugs.

Keywords: Bilayer disks; Drug partitioning; Liposome; Model membrane; PEG lipid; Phospholipid; Immobilized liposome chromatography

1. Introduction

Recent developments in the area of drug discovery, including the use of combinatory chemistry in combination with highly automated synthesis systems, have created a need for methods that allow for fast screening of large sets of drug candidates. Because the majority of drugs have to cross one or several biological membranes in order to reach the target site, evaluation of drug–membrane interactions, and in particular the determination of drug partition and diffusion constants, constitute an important part of the screening procedure. Although predictions obtained by theoretical, in silico, methods [1,2] may serve as valuable tools during the initial selection of drug candidates, there is still a great demand for simple and reliable experimental methods. The validity of these methods relies to a large extent on the availability of suitable model membranes that accurately reflect the physicochemical properties of the relevant biological membranes.

Due to their structural similarity with biological membranes, phospholipid liposomes have been extensively used as model membranes, and during the last 10 years, several liposome-based methods for the determination of drug partitioning have been developed and tested. These include different potentiometric [3–5], chromatographic [6–12], electroforetic [13–15], and calorimetric [16,17] methods, as well as methods based on changes in the spectroscopic behaviour of the drug molecule [18]. The results of these studies indicate the good potential of phospholipid liposomes to serve as models for biological

Abbreviations: DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DPC, drug partition chromatography; EPC, egg phosphatidylcholine; ITC, isothermal titration calorimetry; PEG, poly(ethyleneglycol); PEG-DSPE(5000), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-5000].

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doi:10.1016/j.bpc.2004.09.006

membranes in partition studies. A number of problems have been identified, however, that need to be solved in order to improve the performance and ease of handling of the liposome-based techniques. First, the current methods available for liposome preparation give rise to a significant amount of polydispersity in both the size and structure of the liposomes. Most notably, the preparations do as a rule contain an unknown fraction of bi- or multilamellar liposomes. Depending on the properties of the drug, as well as on the analytical technique used, this fact may give rise to considerable difficulties concerning the interpretation and quantification of the results. More specifically, unless ample time is given for equilibration between the different bilayers of the bi- and multilamellar structures, a proportion of the lipids will, in effect, be hidden from interaction with the analyte. Moreover, also in the unlikely event of a purely unilamellar preparation, the interpretation of data may be confused by the fact that initially only the outer leaflet of the membrane is accessible for direct interaction. Second, because phospholipid liposomes do not represent thermodynamically stable but merely kinetically trapped structures, they tend to aggregate and fuse with time. Eventually, the liposome sample will phase separate into a lamellar phase in excess water. This behaviour is manifested in an inherently poor long-term stability of conventional liposome preparations.

In this study, we introduce an alternative model membrane, the sterically stabilized bilayer disk, which evades the abovementioned problems associated with conventional liposomes.

In addition to their use in drug partition studies, we believe that the sterically stabilized disks may find other important biochemical, biotechnical, and pharmaceutical applications. In particular, the potential use of the disks as carriers for drug delivery deserves attention. A brief discussion on this matter can be found in the Conclusions and future aspects section. In this first communication, we focus, however, on preparation, characterization, and evaluation of sterically stabilized disks intended for drug partition experiments.

2. Materials and methods

2.1. Materials and solutions

Glass columns (HR 5/2 and 5/5 I.D. 5 mm), Superdex 200 prep grade gel and Sephadex G-50 superfine were purchased from Amersham Biosciences (Uppsala, Sweden), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, >99%), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-5000] (PEG-DSPE(5000), >99%), and cholesterol (>98%) from Avanti Polar Lipids (Alabaster, AL, USA), heptyltriphenylphosphoniumbromide and hexyltriphenylphosphoniumbromide from Labora (Stockholm, Sweden), egg phosphatidylcholine (grade 1) from Lipid Products (Nutfield, U.K.), 5- and 6-carboxyfluorescein from Molecular Probes (Leiden, The Netherlands), and alprenolol, atenolol, chlorpromazine, corticosterone, cortisone, hydrocortisone, metoprolol, oxprenolol, pindolol, prednisolone, promethazine, sulfasalazine, verapamil, tetraphenylphosphoniumchloride, and Triton X-100 (reduced) from Sigma (St. Louis, MO, USA). Desmethyldiazepam, diazepam, flunitrazepam, nitrazepam, and oxazepam were gifts from Smith Kline-Beecham (King of Prussia, PA, USA), and diclofenac, diflunisal, flurbiprofen, ibuprofen, indomethacine, indoprofen, lidocaine, loperamide, naproxen, phenytoin, piroxicam, propranolol, theophylline, and warfarin from AstraZeneca (Södertälje, Sweden). Chemicals not listed were of analytical grade or as stated in given references. The buffer used was 150 mM NaCl, 1 mM Na_2EDTA, 10 mM Tris/HCl, pH 7.4.

2.2. Preparation of liposomes and disks

All preparations contained 40 mol% cholesterol and different proportions of DSPC and PEG-DSPE(5000) unless otherwise stated. Cholesterol, DSPC, and PEG-DSPE(5000) were codissolved in chloroform. The chloroform was evaporated under N_2(g), and the remaining chloroform was removed under vacuum for at least 12 h. The lipid film was then hydrated in the buffer at 50 °C and vortexed vigorously. When indicated, the material was extruded 15 times through a polycarbonate filter (100 nm pore size). A LiposoFast membrane extruder from Avestin (Mannheim, Germany) was employed for this procedure.

2.3. Cryo-transmission electron microscopy

Thin (10–500 nm) sample films were prepared by a blotting procedure performed under controlled temperature (+25 °C) and humidity conditions within a custom-built environmental chamber. A small drop of sample was placed on an electron microscopy (EM) copper grid covered with a holey polymer film, and excess solution was removed by blotting with a filter paper. The film was vitrified by plunging the grid into liquid ethane held just above its freezing point, and the grid was thereafter transferred to a Zeiss EM 902 transmission electron microscope (Oberkochen, Germany) for examination. To prevent sample perturbation and the formation of ice crystals, the specimens were kept cool (below −165 °C) during both the transfer and viewing procedures. All observations were made in zero-loss bright-field mode and at an accelerating voltage of 80 kV. The technique has been described in detail elsewhere [19].

2.4. Estimation of entrapped volume

In order to get an estimate of the amount of liposomes present in the disk preparations, we used an assay based on the fluorescence increase due to release, and subsequent dilution of liposome entrapped carboxyfluorescein (CF). A liposome sample containing 5 mol% DSPE-PEG(5000) was

prepared as above but with 100 mM CF included in the buffer. At this concentration, the fluorescence from CF is minimal due to self-quenching. In order to get a homogenous preparation, the liposomes were extruded 15 times through a polycarbonate filter with pore size 100 nm. Nonencapsulated CF was removed on a Sephadex G-50 column, and a portion of the eluted sample was diluted to obtain a total lipid concentration of 15 μM and thereafter transferred to a quartz cuvette. The fluorescence intensity (I_0) was measured on a SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, NJ), with excitation and emission wavelengths set to 490 and 520 nm, respectively. The liposomes were then lysed by addition of Triton X-100 to the final concentration of 4 mM, and the fluorescence intensity was measured again (I_∞). By subtracting I_0 from I_∞, we obtained the intensity value ($I_{5\%}$) corresponding to a pure liposome preparation essentially devoid of disks[1]. The above procedure was then repeated for lipid mixtures including the desired amount of DSPE-PEG(5000). An estimate of the amount of liposomes present in the preparations was obtained by comparing the determined intensity values with $I_{5\%}$.

2.5. Isothermal titration calorimetry

Experiments were performed according to the Solvent–null method described in [17] at 25 °C on a Thermal Activity Monitor (2277 Thermal Activity Monitor, Thermometric AB, Sweden), equipped with a 2-ml titration cell. We measured the free drug concentration as the change in heat flow (dQ/dt in μW) by titrating 20 mg/ml lipid mixed with 10 mg/ml drug with different concentrations of the drug in the sample cell. When the concentration in the cell matched the free concentration in the syringe ($[drug]_{free}$) no heat changes were observed upon mixing. The measurements were corrected for solvent blanks using approximately the free drug concentration in the syringe and the different drug concentrations in the sample cell. The heat generated for lipid dilution was also subtracted. The partition coefficient was calculated according to Eq.(1).

$$K = \frac{[drug]_{bound}}{[lipid][drug]_{free}} \quad (1)$$

2.6. Chromatography on immobilized liposomes and disks

Disks, multilamellar and unilamellar liposomes were immobilized in Superdex 200 prep grade gel beads by rehydrating dried beads with the different lipid suspensions. The material was then packed directly in HR glass columns or, when indicated, first freeze–thawed (−75/+25 °C) five times before packing to entrap more material in the beads. The freeze–thawing procedure is further described in Refs. [20,21]. All columns were equilibrated with the buffer. We applied an analyte (20 μl, 0.1 mg/ml, <5% ethanol) at a flow rate of 0.5 or 1.0 ml/min at 21±2 °C and monitored the elution at 220 nm (267 nm for the phosphonium ions) on Waters 484 or 486 detector (Millipore, MA, USA). Triplicate determinations were performed for all analytes.

The drug partitioning was evaluated from the retention volume, which was expressed as a normalized capacity factor, K_s (M^{-1}) [8].

$$K_s = \frac{V_E - V_0}{A} \quad (2)$$

With V_E, the elution volume of the drug, V_0, the elution volume of an analyte, $Cr_2O_7^{2-}$, that presumably does not interact with the phospholipid bilayers, and A the phospholipid amount. The very small interaction between the drugs and the gel beads can be taken into account as in Refs. [10,11], but have been neglected in this study. We determined the phospholipid content of the gel bed after the analyses by phosphorus analysis [22].

3. Results and discussion

As mentioned in the introduction, the self-closed and often multilamellar nature of conventional liposomes may complicate the evaluation of experimental data obtained in studies of drug–membrane interactions. We anticipated that this problem could be avoided by employing open rather than closed bilayers. Flat bilayer disks, so-called bicelles, can be prepared from a mixture of medium- and short-chain phospholipids [23,24]. Disk-shaped aggregates have been observed also in several other lipid and/or surfactant systems (e.g., Refs. [25–29]). In order to adequately reflect the properties of biomembranes, the disks need, however, to be composed of long-chain phospholipids and should preferably also tolerate incorporation of substantial amounts of cholesterol. Furthermore, many applications require that the size and structure of the aggregates are relatively insensitive to dilution and changes in temperature. The disks found in the abovementioned systems do not meet these demands.

Flat bilayer disks with promising characteristics have, on the other hand, been discovered in dilute aqueous solutions containing a mixture of phospholipids, cholesterol, and polyethyleneglycol lipids (PEG lipids) [30]. PEG lipids are routinely used in order to prolong the blood circulation time of liposomes used for drug delivery applications [31,32]. It is well established that upon inclusion of sufficiently high concentrations of PEG lipids, a transition from bilayer to micellar phase occurs [33,34]. Furthermore, systematic studies of the phase behaviour and aggregate structure in various lipid/PEG lipid systems have revealed that either cylindrical or disk-shaped micelles may

[1] Micrographs obtained by cryo-TEM reveal a, in this context, negligible amount of disks at this concentration.

form as the bilayer saturation concentration is exceeded [30,35]. The cylindrical micelles, which often adapt a long wormlike structure, are observed when PEG lipids are mixed with the unsaturated phospholipid egg phosphatidylcholine (EPC). Disks-shaped structures appear, on the other hand, in mixtures of PEG lipids and saturated lipid analogues like DSPC and DPPC. Importantly, upon inclusion of 40 mol% cholesterol in the lipid mixtures, the PEG lipids induce formation of disk-shaped structures in all the investigated systems [30]. The size of the disks is critically dependent on the PEG lipid concentration. Large disks, perhaps better described as circular membrane patches, are found at PEG lipid concentrations just above the bilayer saturation limit. Investigations based on a combination of dynamic light scattering and cryo-TEM [35] show that the disks are well described by an ideal disk model assuming partial component segregation [35,36]. More precisely, available data strongly suggest that the PEG lipids accumulate at the highly curved rim of the disks, while the phospholipids and cholesterol reside in the bulk of the bilayered aggregates.

We hypothesized that the disks formed in mixtures of phospholipids, cholesterol, and PEG lipids had the potential to work well as model membranes. In order to avoid complications induced by heterogeneous lipid compositions we choose to base our preparations on mixtures of cholesterol and the synthetic phospholipid DSPC. Moreover, the PEG lipid concentration needed for onset of micelle formation decreases with PEG molecular weight [34], and to minimize the amount of PEG lipid in the preparations we therefore opted for a PEG lipid with comparatively long polymer chain, i.e., DSPE-PEG(5000).

3.1. Aggregate structure in mixtures of DSPC, cholesterol, and DSPE-PEG(5000)

Cryo-transmission electron microscopy, cryo-TEM, is ideally suited for direct visualisation of aggregate structure in dilute lipid dispersions [19]. We employed this technique to reveal the morphology in samples containing varying amounts of PEG lipid. Fig. 1 displays the aggregate structure observed in nonextruded samples containing DSPC, 40 mol% cholesterol, and increasing concentrations of DSPE-PEG(5000). As seen by comparing Fig. 1a and b, the inclusion of 5 mol% PEG lipid reverts the sample from a multilamellar to a unilamellar liposome dispersion. Note also how the liposomes due to the polymer-induced steric stabilization appear well separated from each other in Fig. 1b. Micrographs obtained from samples containing 5 mol% PEG lipid reveal, furthermore, a small number of bilayer disks in coexistence with the unilamellar liposomes (indicated with arrows in Fig. 1b and f). As the PEG lipid concentration is increased above 5 mol%, the disks become more frequent and, at the same time, the number of liposomes decreases. A small population of liposomes is still present in samples containing 12 mol% PEG lipid (Fig. 1c), but at higher PEG lipid concentrations liposomes are only rarely observed (Fig. 1d and e).

From a comparison of (Fig. 1c through e), it is clear that the size of the disks decreases with increasing PEG lipid content. When evaluating the actual diameter of the discoidal structures, it is important, however, to realize that the PEG layer cannot be visualized by means of the cryo-TEM technique employed in this study. Due to the very poor contrast between PEG and the vitrified water medium, only the lipid body of the disks is visible to the electron beam. In Fig. 2, the size of the disks, (excluding the PEG layer) as measured from cryo-TEM micrographs, is given for samples containing three different PEG lipid concentrations. The displayed data indicate that the average diameter decreases from about 60 to 25 nm when the PEG lipid content is increased from 12 to 15 mol%. For preparations containing 30 mol% PEG lipid, the average diameter decreases further down to 15 nm. The observation of a PEG-lipid-dependent decrease in the size of the disk is in agreement with earlier conclusions made for mixtures of DSPC and DSPE-PEG(5000) in the absence of cholesterol [35].

Careful visual inspection of the DSPC/cholesterol/DSPE-PEG(5000) samples reveal a somewhat unexpected effect; that is, at high PEG lipid concentrations the mixtures have a tendency to phase separate. Already at 15 mol% PEG lipid, the samples display a vague veil-like turbidity, and with increasing PEG lipid concentration the turbidity becomes more distinct. Above 20 mol% PEG lipid, the samples phase separate with time into a milky white lower phase and a clear upper phase. As shown in Fig. 1e, cryo-TEM investigations reveal for these samples the presence of large irregular bilayer sheets in coexistence with the aforementioned small circular disks. This behavior is in contrast to that observed for the pure DSPC/DSPE-PEG(5000) system where no large bilayer sheets, or indications of a macroscopic phase separation, were discovered [35]. More extensive investigations of the phase behavior in the present and related lipid/PEG lipid systems are needed in order to understand why cholesterol has the effect of promoting the observed macroscopic phase separation.

Based on the above results we concluded that mixtures containing between 12 and 15 mol% PEG lipid were optimal in order to obtain stable preparations dominated by bilayer disks. Because we anticipated that large disks with small edge/body surface area would constitute the most suitable model membranes we choose to use preparations based on 12 mol% PEG lipid in the evaluation studies (see below). As mentioned earlier, cryo-TEM investigations reveal for this composition a small population of unilamellar liposomes in coexistence with the disks. Measurements based on the determination of encapsulated volume (see Materials and methods) indicate, however, that only about 5–10% of the total lipid is situated in closed liposomes.

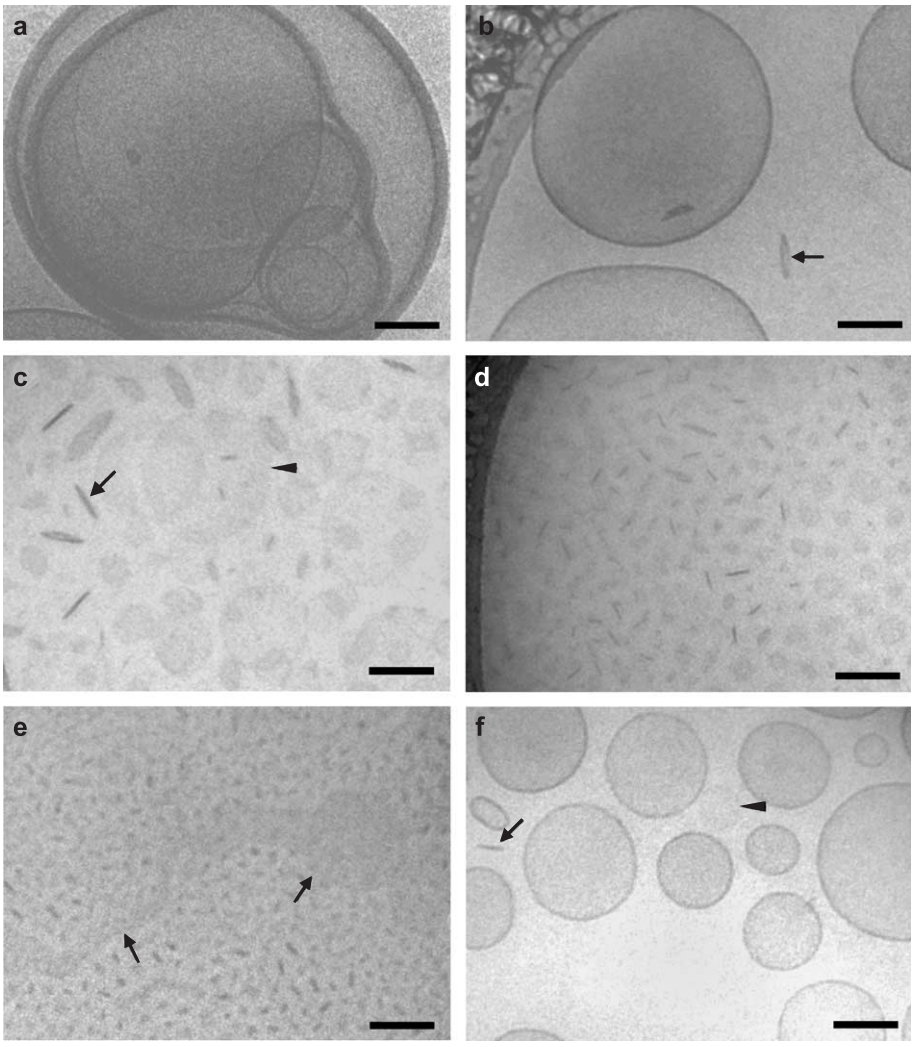

Fig. 1. Cryo-TEM micrographs of DSPC/cholesterol/PEG-DSPE(5000) mixtures with varying amount of PEG-DSPE(5000). Different structures of the lipid bilayer at (a) 0 mol%, (b) 5 mol%, (c) 12 mol%, (d) 15 mol%, (e) 30 mol%, and (f) 5 mol% PEG-DSPE(5000) (extruded). The arrows and arrowheads in (b), (c), and (f) denote disks observed edge-on and face-on, respectively. The arrows in (e) denote a bilayer sheet. Bar, 100 nm.

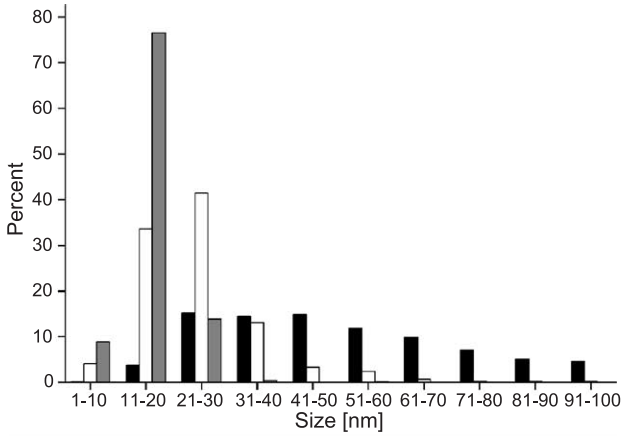

Fig. 2. Size distribution (diameter) of disks measured from cryo-TEM micrographs in DSPC/cholesterol/PEG-DSPE(5000) samples containing 12 mol% (black), 15 mol% (white), and 30 mol% (grey) PEG-DSPE(5000). More than 680 structured counted.

3.2. Effects of preparation path, temperature, and aging

The samples shown in Fig. 1 were prepared by simple vortex dispersion of the hydrated lipid films. Repeated freeze–thawing (including freezing in liquid nitrogen and heating to 60 °C) did not change the size or appearance of the disks noticeably. The disk size remained essentially the same also after repeated extrusion through polycarbonate filters of pore size 100 nm. As expected, the liposome size was, on the other hand, significantly reduced upon extrusion. This fact is illustrated in Fig. 1f, where the aggregate structure in an extruded sample containing 5 mol% PEG lipid is displayed.

Most of the bilayer disks presented to date, such as bicelles and discoidal micelles formed in lipid/surfactant systems, suffer the disadvantage of being very sensitive to changes in lipid concentration and/or temperature. The very low aqueous solubilities of DSPC, cholesterol, and DSPE-

Table 1
Drug partitioning into unilamellar liposomes and disks as analyzed by isothermal titration calorimetry

Drugs and solutes	Charge at pH 7.4	Unilamellar liposomes	Disks
		Log K	
Alprenolol	+	1.22±0.06	1.21±0.04
Lidocaine	+	1.18±0.12	1.10±0.10
Ibuprofen	−	0.85	0.98

Average log K values±S.E.M. for positive drugs of two determinations and log K value of a negative drug.

PEG(5000) render the presently investigated system a comparably low sensitivity to structural changes brought about by dilution. In addition, recently published data indicate that the disks formed in mixtures of DPPC or DSPC and PEG lipids remain stable over a wide range of temperatures [35]. In line with this, we did not detect any changes in the size or structure of the disks when preparations containing DSPC, cholesterol, and 12 mol% DSPE-PEG(5000) were analyzed after incubation at 37 °C for 12 h.

Moreover, the long-term stability of the disk preparations was found to be good: cryo-TEM revealed no significant differences between fresh samples and samples that had been incubated at 4 °C for up to 3 months (results not shown).

3.3. Drug partitioning as investigated by isothermal titration calorimetry

Isothermal titration calorimetry (ITC) constitutes a convenient tool to measure the heat produced or consumed as two liquids of different composition are mixed. Both chemical reactions and noncovalent interactions may give

Table 2
Drug partitioning into multi- and unilamellar liposomes and disks as analyzed by chromatography

Drugs and solutes	Charge at pH 7.4	Multilamellar liposomes[a]	Unilamellar liposomes[b]	Disks[c]
			Log K_s	
Alprenolol	+	1.00±0.00	1.68±0.01	2.13±0.03
Chlorpromazin	+	2.27±0.02	2.86±0.02	3.76±0.11
Heptyltriphenylphosphonium	+	1.26±0.02	1.85±0.07	2.73±0.12
Hexyltriphenylphosphonium	+	1.12±0.02	1.70±0.02	2.31±0.08
Lidocaine	+	0.49±0.10	1.36±0.12	1.27±0.25
Loperamide	+	1.59±0.02	2.30±0.02	3.26±0.09
Metoprolol	+	0.30±0.15	1.34±0.12	1.17±0.12
Oxprenolol	+	0.73±0.01	1.49±0.08	1.67±0.04
Pindolol	+	1.34±0.08	1.85±0.04	2.01±0.08
Promethazine	+	1.84±0.02	2.39±0.04	3.12±0.10
Propranolol	+	1.61±0.02	2.15±0.03	2.63±0.05
Tetraphenylphosphonium	+	1.22±0.03	1.70±0.03	1.99±0.00
Theophylline[c]	+	0.55±0.01	1.31±0.06	1.04±0.38
Verapamil	+	1.19±0.01	1.95±0.01	2.75±0.12
Diclofenac	−	1.74±0.02	2.16±0.06	2.39±0.08
Diflunisal	−	2.26±0.03	2.64±0.08	2.86±0.08
Flurbiprofen	−	1.47±0.01	1.89±0.04	2.03±0.10
Ibuprofen	−	0.53±0.03	1.25±0.04	1.18±0.18
Indomethacin	−	1.63±0.02	2.08±0.06	2.43±0.05
Indoprofen	−	1.07±0.01	1.62±0.00	1.60±0.17
Naproxen	−	1.18±0.01	1.71±0.01	1.73±0.16
Piroxicam	−	1.72±0.01	2.13±0.06	2.26±0.11
Sulfasalazine	−	2.52±0.04	2.89±0.09	3.08±0.10
Warfarin	−	1.54±0.02	1.98±0.05	2.11±0.11
Corticosterone	0	1.12±0.01	1.68±0.00	2.10±0.01
Cortisone	0	1.01±0.00	1.59±0.02	1.79±0.05
Desmethyldiazepam	0	1.81±0.02	2.25±0.06	2.69±0.00
Diazepam	0	1.56±0.02	2.01±0.05	2.42±0.02
Flunitrazepam	0	1.36±0.02	1.83±0.02	2.12±0.04
Hydrocortisone	0	1.03±0.01	1.62±0.00	1.90±0.06
Nitrazepam	0	1.60±0.02	2.06±0.05	2.39±0.02
Oxazepam	0	1.67±0.02	2.13±0.05	2.46±0.02
Phenytoin	0	1.96±0.02	2.37±0.07	2.54±0.14
Prednisolone	0	1.14±0.02	1.67±0.03	1.96±0.05

[a] Average log K_s value±S.E.M. of two columns with multilamellar liposomes immobilized by freeze–thawing.
[b] Average log K_s value±S.E.M. of two columns with unilamellar liposomes immobilized by freeze–thawing. In one of the columns the liposomes were extruded before immobilization.
[c] Average log K_s value±S.E.M. of two columns with disks. The material in one column was immobilized by freeze–thawing.

rise to heat changes, and with modern high sensitivity titration calorimeters, it is, for instance, possible to measure the heat released or consumed due to the insertion of a surfactant into lipid membranes. ITC has previously been successfully used for studies of surfactant-membrane partitioning equilibria [16].

In this study, we employed the ITC technique to determine partition coefficients for a selected set of drugs. As described in the Materials and methods section, we used the so-called Solvent–null method [17], which is based on the injection of a preincubated lipid/analyte mixture into solutions of different analyte concentrations. One advantage of this particular ITC protocol is that the incubation time can be adjusted to assure that true equilibrium is reached during the measurements. The aim of the ITC studies was to perform a comparative investigation of drug partitioning into disks and liposomes, and in this way obtain a first indication as to the potential of the PEG-stabilized disks as model membranes. To ensure that the liposomes employed for the investigations were unilamellar, of uniform size, and did not aggregate during the course of the experiments, we used extruded preparations containing DSPC, 40 mol% cholesterol, and 5 mol% DSPE-PEG(5000) (compare Fig. 1f).

Table 1 displays log K values obtained for the three different drugs, alprenolol, lidocain, and ibuprofen in solutions containing liposomes and disks, respectively. From a comparison of the data, it is clear that the partition behavior is similar in the two different membrane systems. This encouraging result indicates that the varying PEG lipid content in the two model membranes (5 and 12 mol% for liposomes and disks, respectively) has little effect on the equilibrium distribution of the drugs between the water and membrane phases.

Unfortunately the ITC method is limited to drugs having a comparatively high aqueous solubility and is thus not suitable for the vast majority of drugs and drug candidates. Moreover, the experimental setup utilized in this study did not allow investigations of the partition behavior in multilamellar liposomes; the liposomes sedimented quickly and blocked the injection syringe. In addition, investigations of partition behavior by means of ITC are technically demanding and also very time consuming. To overcome the above difficulties, we decided to complement the ITC measurements with investigations of partition behavior using an alternative technique.

3.4. Drug partition chromatography

Per Lundahl et al. [6,7] have developed a technique for partition studies based on immobilization of liposomes in chromatographic gel beads. By means of this method, often referred to as immobilized liposome chromatography or drug partitioning chromatography (DPC), the partition behaviour of large sets of drugs may be quickly and conveniently assessed. The DPC method yields a capacity factor, K_s, that is related but not identical to the partition coefficient K [9].

Log K_s values obtained by DPC for multilamellar liposomes, unilamellar liposomes, and bilayer disks are summarized in Table 2. In addition, Fig. 3 gives a graphic representation of the average log K_s values determined for each of the three systems. From Fig. 3, it is clear that the calculated capacity factors increase according to multilamellar liposomes<unilamellar liposomes<bilayer disks.

Because the unilamellar liposomes and the disks contain 5 and 12 mol% PEG lipid, respectively, it may be tempting to ascribe the observed trend to some general positive interaction between the PEG lipids and the investigated drugs. To rule out this somewhat counterintuitive explanation, we measured and compared the log K_s data obtained for extruded unilamellar liposomes in the absence and presence of PEG lipid. Because liposomes made from DSPC and cholesterol have a tendency to aggregate in the absence of PEG stabilization [26], we based the liposome preparations on egg phosphatidylcholine (EPC) solely. The results of this study show that the inclusion of 4 mol% PEG lipid has no measurable effect on the log K_s values (Fig. 4). Furthermore, the ITC results reported in Table 1 speak against an explanation involving attractive drug/PEG lipid interaction.

In order to understand the reason behind the general trends displayed in Fig. 3, we need to consider and compare the overall structure of the three different bilayer aggregates. As mentioned in the Introduction, the fact that unilamellar and, in particular, multilamellar liposomes only expose a fraction of the total membrane area to the surrounding aqueous media may affect the results of partition measurements. For measurements based on DPC, the presence of a "concealed" lipid fraction may, via overestimation of the accessible lipid amount [A in Eq. (2)], lead to underestimated K_s values. It is important to realize that for preparations containing multilamellar lip-

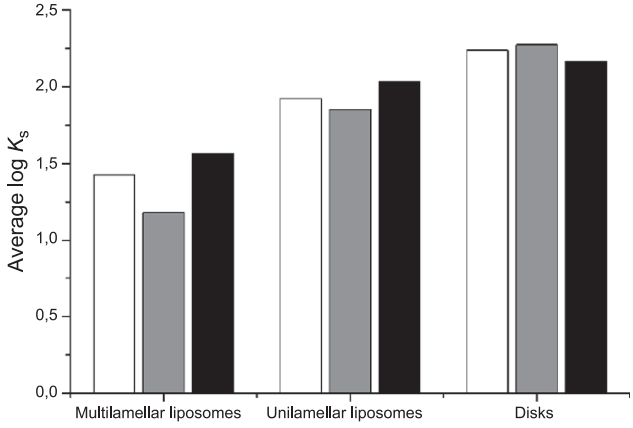

Fig. 3. Drug partitioning for neutral (white), positive (grey), and negative (black) drugs into multilamellar liposomes, unilamellar liposomes, and disks expressed as average log K_s values.

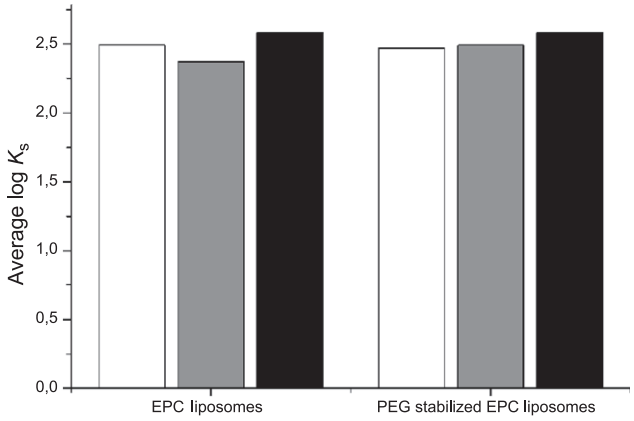

Fig. 4. Drug partitioning for neutral (white), positive (grey), and negative (black) drugs into EPC liposomes and PEG-DSPE(5000)-stabilized EPC liposomes expressed as average log K_s values.

osomes, the potentially inaccessible lipid fraction may be very large.

To what extent the multilamellar structure will affect the outcome of the measurements depends, naturally, on the properties of the drug. Particularly large deviations from the true K_s values are expected for drugs that diffuse slowly over the membrane and thus need long times to equilibrate between the lipid and aqueous phases. Considerable effects may, in addition, be anticipated for drugs that possess a comparably low propensity to partition from the aqueous phase into the membrane. In line with this, the largest discrepancies between the K_s values determined in multi- and unilamellar liposomes are seen for the drugs lidocaine, metoprolol, theophylline, and ibuprofen (Table 2). It should be noted, however, that for all drugs displayed in Table 2, the capacity factor determined in the presence of multilamellar liposomes is consistently lower than that obtained with unilamellar liposomes.

The data displayed in Table 2 (and Fig. 3) show that the average K_s values differ also between unilamellar liposomes and disks. For the majority of the investigated drugs, the calculated capacity factor is significantly higher when determined in the disk system. The difference is, however, not as pronounced as that found when comparing K_s values determined in the multi- and unilamellar liposome systems.

Possible effects caused by electrostatic interactions between the drug and the membrane must also be considered when evaluating the results of the DPC measurements. In contrast to the zwitterionic DSPC molecule, the PEG lipid (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-5000]) used in this study carries a net negative charge. As seen in Fig. 3, where for clarity the calculated average log K_s values for positive, negative, and uncharged drugs are displayed separately, the partition behavior appears indeed to be influenced by the presence of the negative charge. The effects are, as expected, most obvious upon comparison of the capacity factors obtained with disks and multilamellar liposomes, respectively. It is evident that both positively and negatively charged drugs are affected. For the former, the disk system yields disproportionately high K_s values, whereas for the latter suspiciously low values are generated. It is important to note, however, that the general trend concerning the calculated K_s values for the different model membranes, i.e., multilamellar liposomes<unilamellar liposomes<bilayer disks, remains the same irrespective of whether the drugs are positive, negative, or uncharged.

4. Conclusions and future aspects

The results of this study show that aqueous dispersions containing large stable bilayer disks may be produced from lipid mixtures containing DSPC/cholesterol/DSPE-PEG(5000). Initial evaluations by means of ITC and DPC indicate that the disks function well as model membranes in drug partition studies. Moreover, our comparative studies suggest that the membrane disks for certain classes of drugs may generate more accurate data than corresponding measurements based on liposomes.

Some further developments are needed in order to optimize the performance of the disks. First, the disk preparations are fairly polydisperse in size, and strategies to overcome this problem need to be developed and tested. Second, preferably the disks should carry no net charge and preparation protocols based on the use of noncharged PEG lipids are therefore desired. Both these issues are currently being addressed.

Another important aspect concerns the possibility of obtaining sterically stabilized disks with lipid compositions that more closely mimic those of real biological membranes. Our previous observations [30,35,37] indicate that PEG-stabilized disks may be prepared from a variety of phospholipids and phospholipid/cholesterol mixtures. We plan to extend our investigations to explore whether other biologically relevant lipids, such as sphingomyelin and glycolipids, may be included in the disk preparations.

Concomitant with the abovementioned efforts to improve the utility of the sterically stabilized disks as model membranes, we have initiated studies concerning some alternative applications. In particular, we are investigating the potential utility of the disks as membrane mimics for structure/function studies of membrane bound peptides and proteins. For this field of application, the open structure and the low sensitivity to changes in temperature and dilution may render the sterically stabilized disks more attractive than current membrane mimics based on liposomes and classical bicelles. Encouraging results have recently been reported concerning the assembly of functional proteins into nanosized lipid disks composed of phospholipids and specially bioengineered membrane scaffold proteins [38–40]. Widespread use of the nanodisks may however be hampered by the lack of easy access to the scaffold proteins.

Thus, the easy preparation protocols, based solely on commercially available lipid and PEG lipid components, further strengthen the potential of the sterically stabilized disks.

The sterically stabilized membrane disks could, furthermore, find important applications within the field of drug delivery. Bilayer fragments composed of the synthetic lipids dioctadecyldimethylammonium bromide and dihexadecylphosphate have been shown effective in solubilizing the toxic hydrophobic drugs amphotericin B and miconazole [41,42]. Recently published results, furthermore, indicate good in vivo activity for formulations based on this strategy [43]. The hydrophobic drugs are, in this case, believed to accumulate at the edges of the electrostatically stabilized lipid fragments. It is possible that the sterically stabilized disks may be used in a similar way to increase the solubility of poorly soluble drugs. Perhaps even more interesting is the possibility of incorporating hydrophobic drugs in the bilayer interior of the disks. Micelles prepared from pure PEG lipids are comparably stable and can be loaded with a variety of sparingly soluble drugs [44]. Due to the small volume of the micelle hydrophobic core, the solubilization capacity of these polymeric micelles is however rather low. Two recent reports show that the drug solubilization may be somewhat improved by including low concentrations (10–25 mol%) of EPC in the polymeric micelles [45,46]. Attempts to further increase the solubilization capacity by increasing the amount of EPC did, however, result in polydisperse and inhomogeneous preparations. In contrast to the micelles, the PEG-stabilized disks offer large hydrophobic volumes and their size may be tailored to suit different drug delivery applications. A shared advantage of PEG-stabilized micelles and discs is that the drug delivery potential may be enhanced by covalent attachment of targeting agents, such as antibodies or protein ligands, to the distal end of the PEG chains. By use of this strategy, it should be possible to direct the encapsulated drugs to specific organs and tissues in much the same manner as presently being explored for formulations based on PEG-stabilized liposomal carriers [47]. Moreover, the bilayer disks presented in this study have the potential to interact with, and incorporate, a range of amphiphatic drug molecules. We intend to explore the potential of the disks as carriers also for this class of substances. Ongoing studies are, for instance, focused on the development of sterically stabilized disks for controlled formulation and delivery of protein/polypeptide drugs.

Acknowledgements

We are grateful to Göran Karlsson for help with the electron microscopy studies and Anna Lundquist for assistance with the drug partition chromatography. We also like to thank Dan Hallén for help with the ITC equipment. Financial support from the Swedish Research Council, the Swedish Foundation for Strategical Research, the Swedish Fund for Research without Animal Experiments, the Swedish Cancer Foundation, and the O.E. and Edla Johansson Science Foundation is gratefully acknowledged.

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