

DRUG PERMEABILITY ACROSS A PHOSPHOLIPID VESICLE BASED BARRIER

2. CHARACTERIZATION OF BARRIER STRUCTURE, STORAGE STABILITY AND STABILITY TOWARDS pH CHANGES

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Abstract

Recently we reported on the development of a phospholipid-vesicle based barrier as a medium throughput screening method for drug permeability. The aim of the present study was to characterize the barrier structure, including an estimation of the amount of phospholipid within the barrier, its storage stability and stability over a pH range representing the environment in the different part of the gastrointestinal tract. The amount of lipid in the barrier was quantified using a colorimetric phospholipase D-based assay. The total amount was found to be in average 3.30 mg phospholipid in each barrier. The preparation process comprises consecutive deposition of two types of liposomes on a filter support, the smallest liposomes, with a mean diameter of 298 nm, were estimated to fill the pore volume of the filter when tightly packed. The volume of the bigger liposomes, deposited on top of the filter, was calculated to generate a 0.1 mm thick layer. Visualisation of fluorescently labelled liposomes by confocal laser scanning microscopy confirmed that the pores of the filter were completely filled with liposomes and that there is a liposome layer on top. Small angle x-ray scattering (SAXS) analysis was used to study the lamellarity of the liposomes. The liposomes contained oligo- and/or multilamellar structures before and after deposition.

The functionality of the barriers during storage at three different temperatures was examined by measuring the permeability of the hydrophilic marker calcein across barriers stored for up to 4 weeks. The conclusion was that the phospholipid vesicle based barriers could be stored at $-80\text{ }^{\circ}\text{C}$ for up to 2 weeks without significant changes. The stability of the barriers in a pH range from 2.0 to 8.0 was investigated by performing permeation studies with fluoresceine at different pH values. It was found that the phospholipid vesicle based barrier did not loose its integrity within this range. Thus, the barriers appear suitable for further studies to provide insight into segmental absorption in the human gastrointestinal tract. It has also been shown that the barriers can be stored which gives the opportunity to make up larger batches and by

that renders the phospholipid vesicle based barrier more appropriate for larger scale production and high throughput screening.

Keywords: Artificial membrane, liposomes, storage stability, phospholipids, confocal laser scanning microscope, small angle x-ray scattering, pH, phospholipid quantification.

1 Introduction

A novel predictive medium-throughput screening method for passive drug permeability, based on a tight barrier of liposomes on a filter support was recently reported by us (Flaten, et al.,2006). To the best of our knowledge this was the first successful attempt to deposit membrane barriers without the use of an inert solvent such as hexadecane. The phospholipid vesicle based barriers were prepared from egg phosphatidylcholin liposomes, which were deposited into/onto a filter support by centrifugation. Solvent evaporation and freeze-thaw cycling were then used to promote liposome fusion to obtain a tight barrier. The model was validated using 21 drug compounds, which covered a wide range of physicochemical properties and degree of intestinal absorption (F_a) in humans. The apparent permeability coefficients obtained from the phospholipid vesicle based model correlated well with literature data on human absorption *in vivo*, which suggests that its performance is adequate and that the method is suitable for rapid screening of passive transport of new chemical entities (Flaten, et al.,2006). This new approach seems to model the *in vivo* absorption better than simple physicochemical parameters such as PSA and experimentally determined log D, or experimentally more laborious methods such as immobilised liposome chromatography (ILC) (Osterberg, et al.,2001) and the bio-mimetic PAMPA model (Sugano, et al.,2001). The model also appears to function equally well as the Caco-2 model (Yazdanian, et al.,1998) and

the double sink PAMPA (DS-PAMPA) “model” (Avdeef,2003) in the prediction of passive diffusion of drug compounds (Flaten, et al.,2006).

Aqueous phospholipid dispersions of equally high concentration as that of the deposited layer in the current study, have previously been described as “vesicular phospholipid gels” (Brandl, et al.,1997). Such gels have been shown to consist of uni- to oligolamellar bilayer-vesicles in tight packing and to act as a diffusion barrier for incorporated drug compounds (Tardi, et al.,1998). In contrast, in the PAMPA- model, the phospholipid is applied as a solution in organic solvent (Kansy, et al.,1998), which eventually forms monolayers, bilayers or multiple layers at the interface with the water phase. We have hypothesised that the structure of the phospholipid vesicle based barrier consists of liposomal or closed vesicular structures present in the pores and on top of the filter. Since this is a novel method no structural information regarding the barrier has so far been published, so the aim of the present study has been to study the structure of the barrier, including an estimation of the amount and distribution of the phospholipids within the barrier, and to examine the stability of the barrier at different storage conditions and within a pH range from 2.0 to 8.0. Thus, confocal laser scanning microscopy and small angle x-ray scattering (SAXS) were applied for studies of the barrier structure to confirm or modify our hypothesis that the structure of the phospholipid vesicle based barrier consists of liposomal or closed vesicular structures present in the pores and on top of the filter.

So far the phospholipid vesicle based barriers have been made one day before the permeation experiment. To carry this model forward and allow its use in a high- throughput format it was required to study the stability of the barriers during storage. The stability at different storage temperatures has therefore been examined.

The permeation studies performed using the phospholipid vesicle based barrier have been conducted at pH 7.4 in both the donor compartment, the liposome-barrier and the acceptor compartment (Flaten, et al.,2006). However, this pH-value does not fully represent the conditions that drugs encounter during the absorption process *in vivo* in humans. Further studies using different pH conditions in different compartments of the model system are expected to provide insight into segmental absorption in the human gastrointestinal tract. The liposome barrier stability in a broad pH range (pH 2 to 8), representing the environment in the different part of the gastrointestinal tract, has therefore also been examined.

2 Experimental section

2.1 Materials

Egg phosphatidylcholine, Lipoid E-80 was obtained from Lipoid, Germany. Metoprolol tartrate, sulpiride, calcein and cholate were purchased from Sigma-Aldrich Co, St. Louis, USA. Fluorescein sodium was supplied by Merck KGaA, Darmstadt, Germany. LissamineTM rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Rh-DHPE) was obtained from Molecular Probe, Eugene, USA. Silver behenate was supplied by NIST, Gaithersburg, USA.

Culture inserts (Transwell-Clear, d = 6.5 mm) and plates were purchased from Corning Inc, Corning, USA, the filter removed and replaced by mixed cellulose ester filters (0.65 µm pore size) from Millipore, Billerica, USA. The phospholipid kit “Phospholipids B-enzymatic colorimetric method” was purchased from Wako Chemicals, Neuss, Germany.

2.2 Methods

2.2.1 Preparation of the barrier

The phospholipid vesicle based barriers were prepared according to the procedure described earlier (Flaten, et al.,2006) unless stated otherwise. In brief, liposomes with two different sizes were deposited on a filter support in consecutive steps, first the smaller liposomes and then the larger, by use of centrifugation. Freeze-thaw cycling was then used to promote liposomes fusion to produce a tight barrier.

2.2.2 Structure of the barrier

2.2.2.1 Confocal laser scanning microscopy

The liposome barriers were prepared as described earlier (Flaten, et al.,2006), with the exception that 0.2 mol% of the egg phospholipids was displaced by the fluorophore Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-DHPE) (Kirjavainen, et al.,1996). The barriers were hydrated in calcein solution (0.165 mg/ml) (Nagahara, et al.,2004) in phosphate buffer (pH 7.4). The incubations were performed by adding calcein solution to the donor and the acceptor chambers, and allow the system to equilibrate for 3 hours. A mixed cellulose ester filter was also incubated in calcein solution (0.165 mg/ml) to enable the use of the filter without the liposomes as a reference. The barriers and filters were gently removed from the inserts by use of a scalpel and placed on a microscope slide (Menzel-Glaser, Menzel GmbH + Co KG, Braunschweig, Germany). The fluorophores were stabilized by addition of Fluoromount-G (SouthernBiotech, Birmingham, England) and the barrier was covered with a cover slip (Menzel-Glaser, Menzel GmbH + Co KG, Braunschweig, Germany).

The sample was examined under a confocal laser-scanning microscope (Leica DM IRE2 from Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). Rh-DHPE and calcein were excited by use of lasers with wavelength 543 nm and 488 nm, respectively. The emission spectra had their maxima at the spectral maxima for calcein and Rh-DHPE, 509 nm and 580 nm, respectively.

2.2.2.2 Amount of phospholipids in the barrier

Barriers were investigated both after the deposition of only the smaller liposomes to enable quantification of the amount of phospholipids in the pores of the filters, and after completed preparation in order to quantify the total amount of lipid deposited. The inserts with the barriers were incubated in a cholate solution (2 mg/ml) (5 ml) under bath sonication for 45 min to solubilize the phospholipids in the barrier. The mass balance of the phospholipid dispersion was estimated by quantification of the amount of phospholipids not caught in or on the filter support as well, and comparing it to the overall amount applied during preparation (18 mg per filter insert). The liposome dispersion passing through the filter during centrifugation (Flaten, et al.,2006) was also collected and analysed upon solubilization with cholate. Quantification of phosphatidylcholine was carried out as described in (Grohgan, et al.,2003) with the following adaptations: 50 µl of the samples and 250 µl of the phospholipid B-enzymatic colorimetric reagent were mixed in a 96-well titer plate and incubated for 45 min in the titer plate reader at 37 °C, with shaking for the first 5 minutes. The UV-absorption at 492 nm was measured and evaluated against standard solutions of E80 and cholate. The experiments were done in triplicate with four inserts in each parallel. The mean values and standard deviations are reported.

2.2.2.3 Liposome size analysis

The size of the liposomes was measured by photon correlation spectroscopy (PCS) using a Submicron Particle Sizer 370, (PSS Nicomp Particle Sizing Systems, Santa Barbara, California, USA). Sample preparation and measuring conditions were as described earlier (Ingebrigtsen and Brandl, 2002). Three cycles with 10 min measuring time each were performed.

The total number of particles with a diameter larger than 0.5 μm , in the liposome dispersion extruded through filters with pore size 800 nm, was also estimated by optical particle counting using a AccuSizerTM 780 Optical Particle Sizer (PSS Nicomp Particle Sizing Systems, Santa Barbara, California, USA). The liposome dispersion was diluted extensively ($1:1.66 \times 10^{-6}$) with particle free water until a concentration for which the particle counter could detect single particles in the flow line. The number of particles in the lipid dispersion was then calculated back from the total dilution and the measured particle number. Three parallels were performed and the results represented the combined results from three 5 ml aliquots.

2.2.2.4 Small angle x-ray scattering (SAXS)

The samples were investigated with a Kratky camera (MBG-SAX, Hecus M. Braun, Optical Systems GmbH, Graz, Austria) with a Iso-Debyeflex 3003 60 KV generator (Rich. Seifert, Ahrensburg, Germany), an x-ray tube (copper anode) FK 61-04 x 12 and a position sensitive detector (PSD-50M, M. Braun, Garching, Germany). For a simultaneous small and wide angle scattering measurement the wide angle extension of the device (MBG-SWAX) containing an additional detector for the wide angle range was used. In most cases, the samples were measured for one hour at 25 °C (30 min for the SAXS investigation of the E80 raw material). Hand-shaken liposome dispersion and liposome dispersions extruded through filters with pore sizes 800 nm and 400 nm were examined. Also a mixed cellulose ester filter

wetted in phosphate buffer and phospholipid vesicle barriers both before and after incubation in phosphate buffer for 3 hours were studied using a sample holder for semisolids. The three liposome dispersions and the buffer were also examined in a capillary sample holder to allow a more exact comparison of the reflection intensities. The small angle reflections of silver behenate ($d_{001} = 5.838$ nm (Gilles, et al.,1998,Hung, et al.,1993)) were used to calibrate the s-axis. After addition of phosphate buffer changes of the barrier structure during extended incubation in buffer at 25 °C were followed first by measurements at 5 min intervals for 30 minutes, followed by continuous analysis over 5 hours at 15 minutes intervals (5 hours is the normal time period for a permeation experiment). The measured scattering curves were smoothed and desmeared to obtain the data presented in Figure 5-7.

2.2.3 Storage stability measured by permeation of calcein and electrical resistance

One exception was made in the preparation procedure of the barriers stored at -80 °C. Here the last step in the barrier preparation procedure, heating at 65 °C for 30 minutes, was not done until the same day as the permeation studies were performed. The barriers were stored at three different temperatures; 20 °C, 2-8 °C or -80 °C. The storage times were 1 and 2 weeks, barriers were also stored at -80 °C for 3 and 4 weeks.

Permeation studies were performed using 95.5 mM calcein solutions in phosphate buffer (pH 7.4) according to the procedure described earlier (Flaten, et al.,2006). At the end of the permeation experiment, samples (200 µL) from each acceptor compartment were transferred into 96-well black plates (Costar) and analyzed at 28.5 °C in a Polarstar (Fluostar, BMG Technologies) fluorimeter with excitation and emission wavelengths at 485 and 520 nm, respectively. The resistance of the lipid barriers was measured (Millicell-ERS, Millipore, USA) immediately after completion of permeation studies. A value of 119 ohms resulting

from the filters was subtracted from the observed resistance to account for the resistance resulting from the lipid barrier alone. The obtained value was multiplied by the surface area (0.33 cm^2) to normalize for the dimensions of the insert. The experiments were performed at least in triplicate with six inserts in each parallel for each storage condition and storage length. The mean values and standard deviations are reported.

2.2.4 Barrier stability towards changes in pH in a range from 2.0 to 8.0

Permeation studies were performed with fluoresceine solutions in phosphate buffer (pH 2 and 8) according to the procedure described (Flaten, et al.,2006). In the acceptor well a phosphate buffer (pH 7.4) was used. The analysis of the samples and the measurement of the electrical resistance were done as described in section 2.2.3. The experiments were performed at least in triplicate with six inserts in each parallel for each pH. The mean values and standard deviations are reported.

3 Results and discussions

In this study the structure of the phospholipid vesicle based barrier should be investigated in more detail. In order to do so firstly, the distribution of differently fluorescently labelled lipid and water phases over the depth of the filter support was visualised using confocal laser scanning microscopy. Furthermore the size of the smallest liposomes used was measured by PCS and the amount of phospholipid which had been deposited during the first two centrifugation steps determined. This allowed estimation of the volume occupied by the liposomes in tight packing compared to the pore volume of the filter. Secondly, the amount and size of the bigger liposomes deposited during the subsequent centrifugation step was measured and the volume occupied by the liposomes in tight packing estimated in terms of

thickness of the liposome layer on top of the filter. Thirdly, the contribution of each of the two liposome layers in the barrier with regard to solute permeability and electrical resistance were determined. In addition, the structure of the different types of liposomes prior and after deposition on the membrane was investigated by small angle x-ray scattering.

Finally, the stability of the barriers at different storage temperature (+20 °C - -80 °C) and within a wide pH range (pH 2-8) have been examined.

3.1 Structure of the barrier

Our hypothesis regarding the structure of the phospholipid vesicle based barrier implicates that liposomal or closed vesicular structures are present in the pores as well as in a layer on top of the filter (see Figure 1). This assumption is supported by literature data, e.g. extrusion of hydrated phospholipids has earlier been shown to yield liposomes of a size corresponding to the pore size of the filters used (Berger, et al.,2001). However, the use of repeated extrusion cycles through the same filter pore size has been shown to reduce the liposome size (Hope, et al.,1993). The presence of ethanol in the hydrated phospholipids, in the concentrations used in this study, has also been shown not to disturb liposome formation (Polozova, et al.,2005). The barriers were made by first adding smaller liposomes which are expected to go into the pores, and then larger liposomes that are supposed to produce a layer on top of the filter.

Figure 1

3.1.1 Confocal laser scanning microscopy

To study the phospholipid vesicle based barrier in a confocal laser scanning microscope the liposome barrier was visualized using rhodamine, a red fluorescent marker, and the buffer using calceine, a green fluorescent marker. Rhodamine ethanolamine has been widely used in

studies of membrane fusion, ion permeation and lipid transfer between vesicles. It is not dependent of pH changes or molecular aggregation in the concentration used in this study (Massari, et al.,1988).

The results from the confocal laser scanning microscopy are shown in Figure 2 where cross-section pictures of a phospholipid vesicle based barrier (A and B) and a plain mixed cellulose ester filter (C) are shown.

Figure 2.

Figure 2 shows that the barrier consists of lipid filled pore structures as well as a liposome layer on top of the filter. In Figure 2C it can be seen that the pore structures of the mixed cellulose ester filter are filled with calcein solution. In Figure 2A and 2B the pores appear to be filled with liposomes through the whole filter thickness. No transient aqueous channels could be observed in the pictures. In some spots on the filter surface the liposome layer appeared to be thinner or almost absent, but since the pictures also show that the pores are filled with liposomes there are still no aqueous channels going through the whole barrier. The tightness of the barriers, also after incubation with buffer for 5 hours, is comparable with that observed by Brandl et al (Brandl, et al.,1997), where vesicular phospholipid gels are reported to make up a tight barrier towards water soluble markers and Tardi et al (Tardi, et al.,1998) where no floating away of liposomes from the vesicular phospholipid gel was observed, at least for phospholipid gel containing multi-lamellar structures and planar bilayer staples, for weeks.

3.1.2 Amount of phospholipids in the barrier

To further confirm the barrier structure seen in the microscopy studies, the amount of phospholipid in the barrier was quantified. First the total amount of phospholipid in each barrier, as well as the distribution between the amount of phospholipids originating from the smallest liposomes in the pores and the larger liposomes expected to be layered on top of the filter, was determined. This was done by dissolving the barrier phospholipids in a cholate solution by sonication and then quantifying the amount of egg phosphatidylcholine both in half-finished (after the addition of only the smallest liposomes) and finished barriers (after also the addition of the larger liposomes) using a colorimetric phospholipase D based serum-phospholipid assay (Grohgan, et al.,2003). The size distribution of the different liposome dispersions was also determined to allow an estimation of the volume that the liposomes theoretically would fill.

The quantification of the amount of egg phosphatidylcholine in the half-finished barriers gave a mean value of 0.5 ± 0.2 mg phospholipids per barrier. The analysis of the size distribution of the liposomes by PCS showed that the liposomes extruded through filters with a pore size of 400 nm had a volume-based mean diameter of 298 ± 7 nm. In Figure 3 an example of the volume based size distribution in the liposome dispersion is given.

Figure 3.

When assuming that each egg phospholipid molecule covers a surface area of 71.7 \AA^2 (Lasic,1993) and that the liposomes are unilamellar it is possible to make a theoretical calculation of the overall volume of the liposomes. This again allows an estimation of how much of the pore volume of the filter that is filled with liposomes. When comparing the overall volume of the 298 nm-liposomes with the overall pore volume, it was found that there

are more liposomes present than the amount corresponding to the densest sphere packing (74% of the total volume, (Kleber,1985). The liposomes are not rigid globes, they will to some extent squeeze together. In addition, it is not certain that all the small liposomes go into the pores; some could be stacked on the surface of the filter when the pores have been filled. Another point that has to be taken into account is that the liposomes are assumed to be unilamellar when doing these calculations. Oligo- or multilamellar structures will decrease the volume that a specific amount of phospholipids would fill. So what could be said from these calculations is that the pores of the filter are more or less completely filled with liposomes. This is also in agreement with the result obtained from the confocal laser scanning microscopy.

After the addition of the larger liposomes a quantification of the total amount of egg phosphatidylcholine in the finished barrier was performed using the same method. A mean value of 3.3 ± 0.2 mg phospholipid was found in each barrier. Subtracting the amount of phospholipid originating from the smaller liposomes, already known to be 0.5 ± 0.2 mg, gives 2.8 mg phospholipids originating from the larger liposomes extruded through filters with pore size 800 nm. The overall recovery of liposomes was close to 100%. For these larger liposomes the PCS method appeared not to deliver reproducible results, so the size distribution was instead estimated by optical particle counting. The number weighted mean diameter was estimated to be 607 ± 6 nm. In Figure 4 the size distribution of the liposome dispersion is shown. By calculating the volume of these liposomes in the same way as for the smaller liposomes and dividing this volume by the area of the filter, the thickness of the liposome layer was found to be 0.1 mm.

Figure 4.

The amount of lipid on the filter mainly originates from the addition of liposomes extruded through filters with a pore size of 800 nm. As much as 86% of the lipids in the barrier originate from this step in the preparation procedure, whereas 14% originate from the two additions of liposomes with a mean diameter of 298 nm. To investigate how much the lipids in the pores contribute to the barrier properties, barriers were made using only liposomes extruded through filters with a pore size of 800 nm. The permeability (P_{app}) of calcein across this barrier was found to be $0.378 \pm 0.064 \times 10^{-6}$ cm/sec and the electrical resistance measured was $660 \pm 118 \Omega$. For the barriers made by addition of both liposomes extruded through filters with pore size 400 nm and liposomes extruded through filters with pore size 800 nm these values was $0.061 \pm 0.005 \times 10^{-6}$ cm/sec and 1591Ω , respectively. This shows that the phospholipids in the pores contribute significantly to the permeability properties of the barrier.

3.1.3 Small angle x-ray scattering (SAXS)

To study the liquid crystalline structure in the different liposome dispersions and the barrier, as well as to look into the hydration process of the barrier, SAXS experiments were performed.

The scattering profiles of both the hand-shaken dispersion and the liposome dispersions extruded through 400 nm and 800 nm filters, respectively, all display two diffraction signals with a spacing ratio of 1:2 characteristic of lamellar arrangements (Figure 5). The d-spacing of the lamellae was found to be about 6.3 nm for the extruded liposomes and about 6.5 nm for the hand-shaken liposomes which is in good agreement with the value previously reported for fully hydrated egg lecithin (6.4 nm (Small,1967)). A comparison of the signal intensities reveals that the amount of lamellar structures decreases with increasing dispersion of the

samples. There is, however, still a considerable degree of lamellarity even in the dispersion extruded through the 400 nm filter (structures composed solely of single bilayers would display only diffuse scattering without any sharp signals (e.g. (Westesen and Wehler,1992))).

Figure 5.

Also the SAXS curves of the fully hydrated membrane display sharp diffraction signals indicative of multilamellar arrangements with a d-spacing value (~ 6.6 nm) close to that of the hand-shaken liposomes (Figure 6). The signals arising from the fully hydrated lipid membrane are, however, distinctly sharper than those from the liposomal dispersion which might indicate a higher degree of order and/or a larger size of the lamellar domains in the membrane. This thus seems to resemble the morphology detected in vesicular phospholipid gels by freeze fracture electron microscopy (Brandl, et al.,1997). The position of the reflection arising from the partially hydrated lipid barrier corresponds to a much smaller interlamellar distance (~ 5.1 nm; reflections of 2nd and 4th order were also observed but only be viewed at much higher magnifications) resulting from dehydration. Similar d-spacing values have been reported for egg lecithin containing $\leq 15\%$ water (Small,1967). Comparisons with these values have, however, to be done cautiously due to the use of different sources of the egg lecithins in the investigations. The loss of solvent during membrane preparation is, however, not high enough to convert the lecithin back to its original state which is characterized by a more complex scattering pattern. The egg lecithin raw material is at least partially crystalline as indicated by the occurrence of reflections in the wide angle range.

Figure 6.

The hydration process of the barrier was examined by time resolved SAXS measurements.

The strong reflection of the partially hydrated lipid barrier corresponding to ~ 5.1 nm shifted

to much smaller angles within the first minutes after addition of buffer (Figure 7). The hydration process continued for about one hour with decreasing velocity. After this time the signal remained more or less constant.

Figure 7

In summary, our hypothesis regarding the barrier structure has to a large extent been supported by the studies presented above. The cross-section pictures from the confocal laser scanning microscope showed that the pores are filled with liposomes and that there is a liposome layer on top of the filter. The quantification of the amount of phospholipids in the pores and on the surface of the filter also supports this hypothesis. The results from the x-ray scattering analysis show that there are oligo- and/or multilamellar structures.

3.1.4 Storage stability measured by permeation of calcein and electrical resistance

The stability of the barriers during storage at three different temperatures was verified by performing permeation experiments using calcein after different storage times. The mean values and standard deviations of the permeability (P_{app}) of calcein obtained from the barriers stored under the different temperatures are given in Figure 8. Only small changes in the electrical resistance ($\pm 100 \Omega \times \text{cm}^2$) were observed.

Figure 8.

As can be seen from the results in Figure 8, storage at -80°C is the most promising condition. The barriers can be stored up to two weeks under these conditions without any significant changes with respect to permeability of calcein and electrical resistance. After storage for more than two weeks an increase in the permeability of calcein can be observed, but neither the standard deviation for the permeability nor the electrical resistance was changed. The

barriers stored at 20 °C showed a larger change with time in both calcein permeability and electrical resistance. The mean value for the calcein permeability for barriers stored at 2-8 °C was not changed with prolonged storage time, but the standard deviation was increasing dramatically which means that the barriers are not stable for longer time under these conditions.

3.2 Barrier stability towards changes in pH in a range from 2.0 to 8.0

The stability of the phospholipid vesicle based barriers in a pH range from 2.0 to 8.0 was studied by performing permeability experiments with fluorescein at different pH values in the donor compartment. The mean and standard deviations of the permeability (P_{app}) of fluorescein at the different pH values are given in Figure 9. The electrical resistance measured after completion of the experiment showed no significant changes.

Figure 9.

The fluorescent marker used in these experiments, fluoresceine sodium, was chosen as its charge is not changed within the pH range used (pK_a 8.6)*. The phospholipid vesicle based barriers seem to be stable according to the permeability of fluoresceine over the pH range examined (Figure 9). Neither the electrical resistance values were significantly changed within this pH range. Thus, the barriers do not seem to loose their integrity in the pH range from 2.0 to 8.0.

* The value is calculated using Advanced Chemistry Development (ACD/Labs)

4 Conclusions

In this study we have investigated the structure of the phospholipid vesicle based barriers as well as their stability at different storage temperatures and within a wide pH range.

The studies of the structure of the barrier support our hypothesis that the pores of the filter are filled with liposomes and that there is a layer of liposomes on top of the filter. There are oligo- and multilamellar structures and most probably also unilamellar structures in the barriers. The phospholipid vesicle based barriers are stable at $-80\text{ }^{\circ}\text{C}$ for up to two weeks and can stand the pH range from 2.0 to 8.0 without losing their integrity.

As the barriers have a structure that is stable under a wide pH range they are suitable for permeation studies using different pH conditions in different compartments of the model system. Such a setup is expected to provide insight into segmental absorption in the human gastrointestinal tract. The satisfying stability at the preferred storage temperature also gives the opportunity to produce larger batches of the barriers and by that make the laboratory work more efficient and the phospholipid vesicle based barrier more appropriate for medium to high throughput screening.

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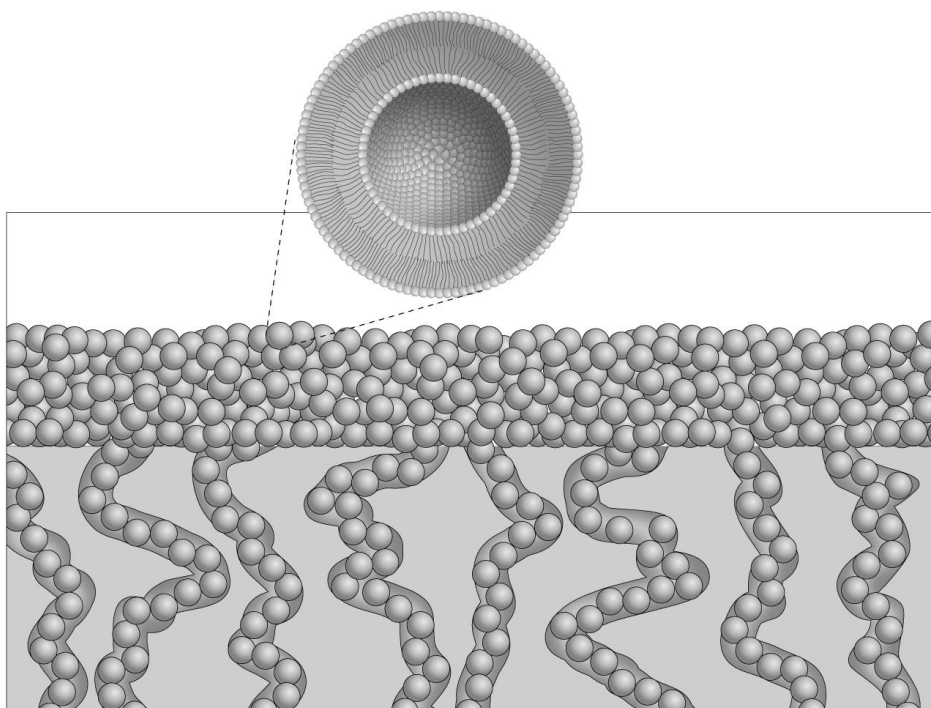


Figure 1. A schematic illustration of the hypothesised arrangement of the phospholipid vesicle based barrier.

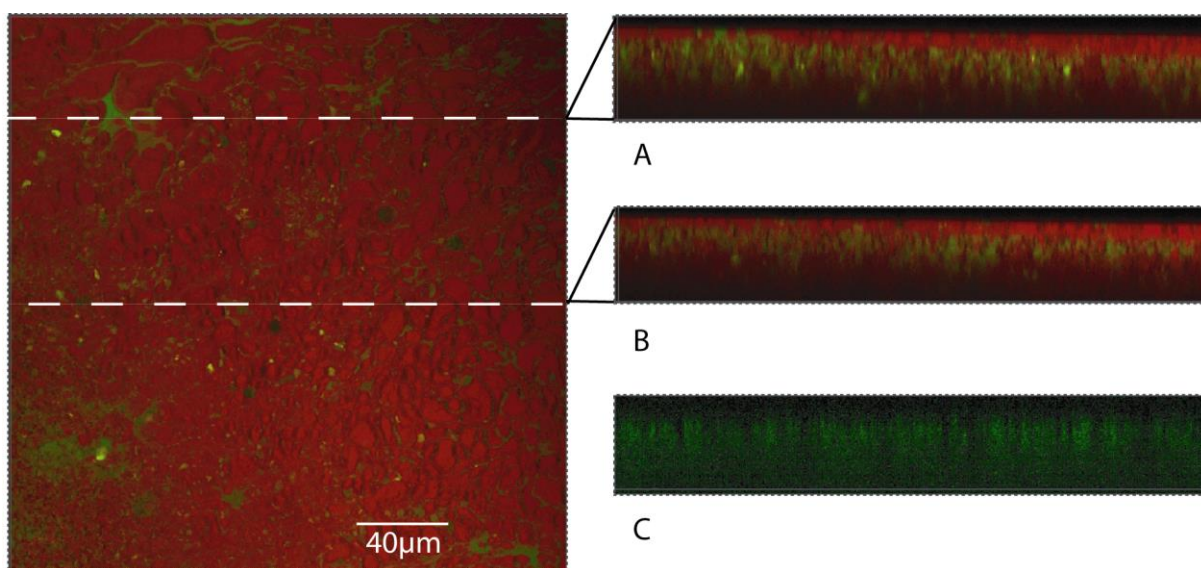


Figure 2. Cross-sections from the confocal laser scanning microscopy studies of the phospholipid vesicle based barrier (A and B) and the mixed cellulose ester filter (C). Microscopy pictures from different levels in the membrane are given in Figure A in the Supplementary Material

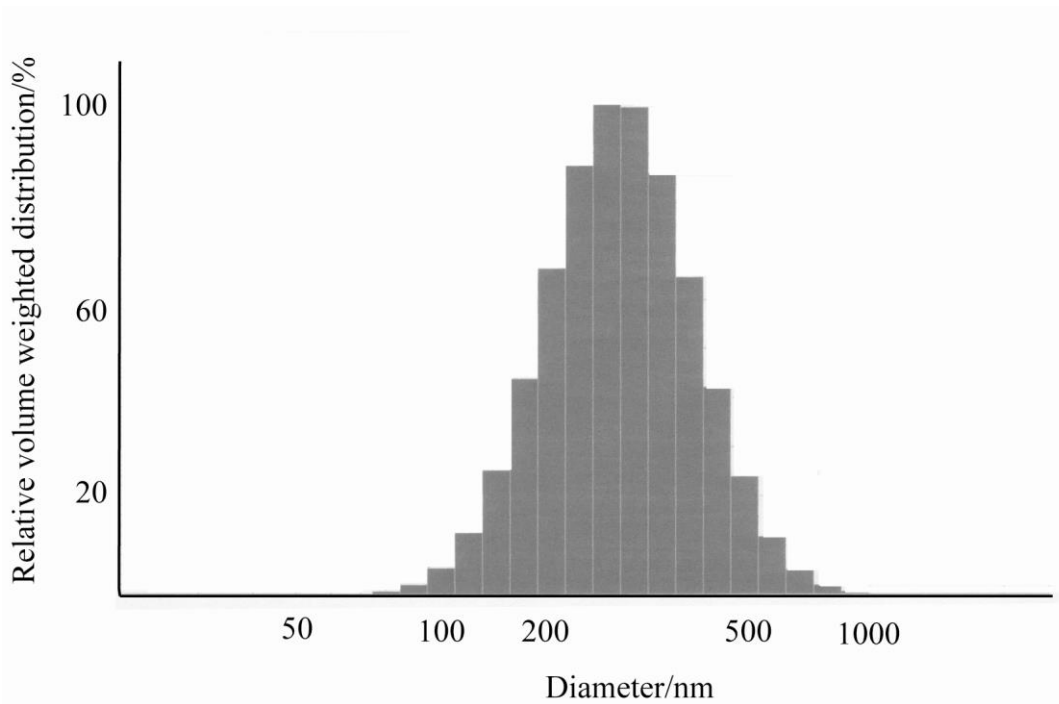


Figure 3. Size distribution of the liposomes extruded through filters with pore size 0.4 μm , as obtained by PCS (volume-weighted).

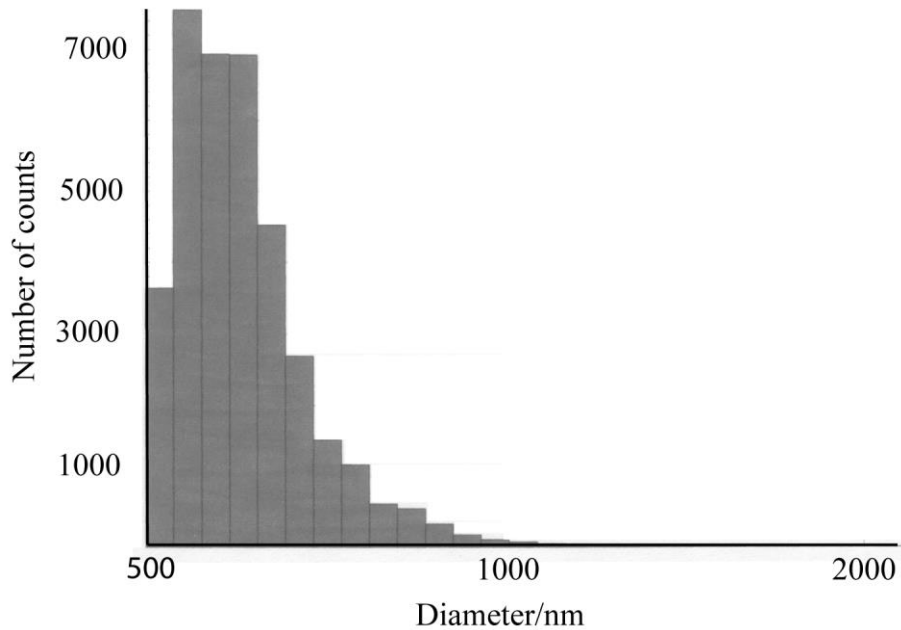


Figure 4. Size distribution of the liposomes extruded through 800 nm filters, as obtained by light obscuration particle counting.

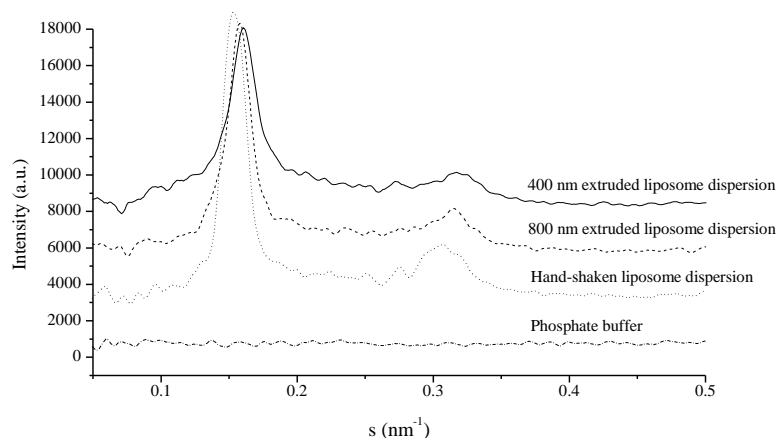


Figure 5. SAXS profiles of the three liposome dispersions and the phosphate buffer (samples were investigated in a capillary sample holder to ensure equivalent sample thickness). The curves are displaced along the ordinate for better visualization. ($s = 1/d = 2 \sin \Theta / \lambda$ where 2Θ is the scattering angle and λ is the wavelength)

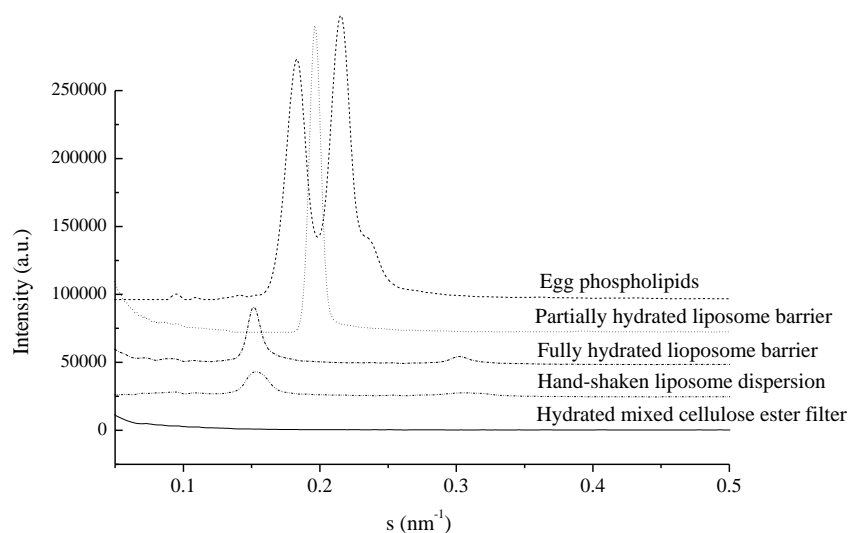


Figure 6. SAXS curves of the partially hydrated barrier, the fully hydrated barrier, the hydrated mixed cellulose ester filter, the hand-shaken liposome dispersion and the egg lecithin. The curves are displaced along the ordinate for better visualization.

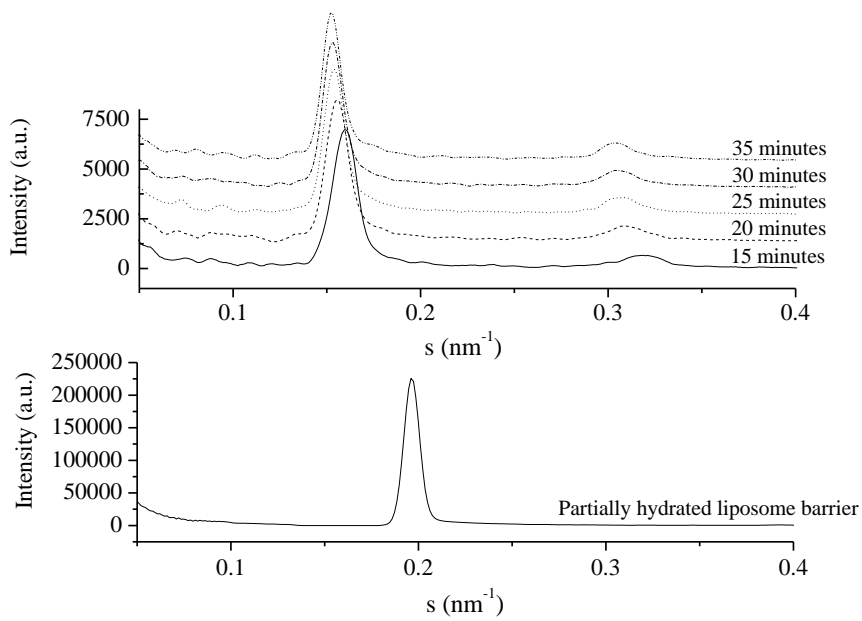


Figure. 7: SAXS curves of the lipid barrier observed prior (bottom) and at certain time points after addition of phosphate buffer to the barrier (top). The curves are displaced along the ordinate for better visualization.

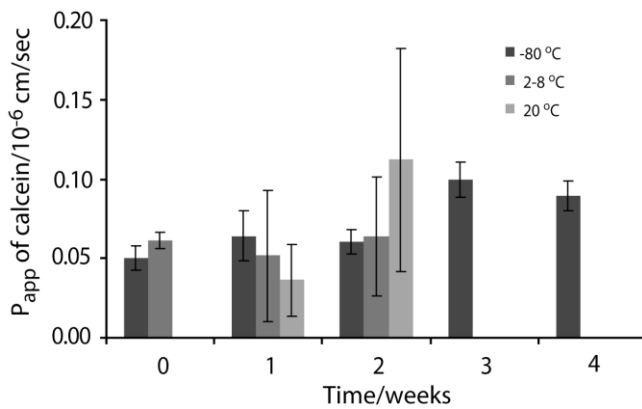


Figure 8. The permeability of calcein versus storage time at -80 °C, 2-8 °C and 20 °C, respectively. The 0 week results were obtained from barriers stored for 18 hours at the given temperature.

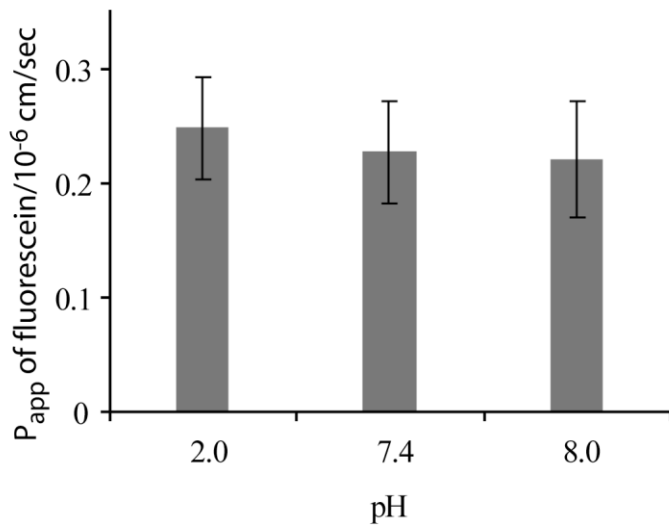


Figure 9. The permeability (P_{app}) values of fluorescein using different pH-values in the donor compartment. In the acceptor compartment the pH was 7.4