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PAPER

Covalent binding of phospholipid vesicles on fused silica capillaries for electrochromatography

Jana Lokajová,[a] Heidi Tiala,[a] Tapani Viitala,[b] Marja-Liisa Riekkola[a] and Susanne K. Wiedmer*[a]

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A rapid method for covalent binding of phospholipids with primary amino groups on fused silica capillaries was developed. In the 8 hour coating procedure the reaction product of aminopropylsilylation of the silanol groups of the fused silica capillary reacts with glutaraldehyde giving an imidoaldehyde which then reacts with the primary amino group of the phospholipid. Various types of liposomes with a broad range of composition and concentration were tested, and the role of primary amino groups in lipids was clarified. Since only 2.5% of lipids with primary amino groups were needed for stable covalent binding of liposomes to the fused silica capillary wall, the method allowed fine-tuning of the lipid composition to mimic given *in vivo* conditions. The optimized coating showed good stability, with intra-day and inter-day repeatability of electroosmotic flow equal to 2.36% RSD ($n = 15$) and 3.56% RSD ($n = 3$), respectively. The capillary to capillary reproducibility was 5.66% RSD ($n = 3$). The thickness and other properties of the attached liposome layer were measured using quartz crystal microbalance (QCM) technique. The capillary surface with covalently attached liposomes acted as a high throughput assay for the determination of interactions between the phospholipid bilayer and the aqueous phase. A model set of structurally diverse drugs was separated in the liposome coated capillary. The retention factors and distribution values were used as indicators of affinity of the drugs toward liposomes.

Introduction

The number of new possible therapeutic drugs is rising, following rapid scientific advances in human genomics and proteomics. Accordingly, high-throughput screening methods for predicting analyte interaction with biomembrane of various compositions are needed. The octanol–water partition coefficient (log *P*) is still today the most common measure for evaluating drug–lipid membrane interactions. Even though it provides the description of lipophilic properties, it fails in mimicking the biomembrane environment, mostly because it considers only non-polar interactions, see ref. 1 and 2 and citations therein. The other widely used method for predicting interactions between drugs and membranes or proteins is equilibrium dialysis.[3,4] The main challenge in equilibrium dialysis measurements is no longer the liquid volumes but more the time needed for reaching the equilibrium state—rapid equilibrium dialysis requires at least 100 min and vigorous agitation—as well as the correct selection of semi-permeable membranes.

Among other principal experimental approaches used for high-throughput determination of drug–membrane interactions is affinity capillary electrophoresis.[5,6] The retention of solutes in this method is based on the similar interactions found in biological systems. In electrokinetic chromatography (or affinity capillary electrophoresis) the ligand is part of the background electrolyte solution. Such a setup requires a large amount of ligand, which increases analysis costs. On the other hand, immobilization of the ligand in capillaries only needs low amounts of ligand, which is beneficial for high-throughput analyses.

The main disadvantage of commercial immobilized artificial membrane chromatography phases is that currently there is only phosphatidylcholine (PC)-based stationary phases available and they are very expensive. PC is by far the most common lipid in biological membranes, however, it poorly represents biological membranes because of its zwitterionic character (only slightly negatively charged). Instead, most biological membranes are negatively charged and have complex lipid compositions.

The formation of supported phospholipid bilayers on various types of materials has gained extensive attention over the last few years, see *e.g.* ref. 7–11 and references therein. We have focused much in our group on dynamic (physically adsorbed) lipid coatings for open-tubular capillary electrochromatography (CEC) in studies on interactions between lipid membranes and

[a]*Laboratory of Analytical Chemistry, Department of Chemistry, POB 55, FIN-00014 University of Helsinki, Finland. E-mail: susanne.wiedmer@helsinki.fi; Fax: +358 9 191 50253; Tel: +358 9 191 50264*
[b]*Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, POB 56, FIN-00014 University of Helsinki, Finland*

analytes.[12–18] The dynamic, or physically adsorbed, lipid coating methods are fast and provide good flexibility in the composition of liposomes to mimic (biological) biomembrane lipid bilayers. The lipids in the form of bilayer membranes or lipid vesicles are dynamically attached on the inner surface of silica capillary by a simple procedure of rinsing the capillary with the lipid solution. The dynamic lipid coatings can be reproducibly manufactured. However, unfortunately especially limited stability has hindered the dynamic lipid coatings from being useful in CEC with mass spectrometric detection. Another drawback is the low capillary column lifetime which suppresses their application in quantification of interactions between compounds and liposome or phospholipid membranes.[19]

Another technique to immobilize phospholipids on silica capillaries is to use avidin–biotin coupling. Avidin is a tetrameric protein, which binds biotin with a high degree of affinity,[20] and specificity. It enables the formation of high-order liposome aggregated by the site-specific self-assembly process.[21] The biotinylated liposomes have been immobilized on the capillary wall which is preconditioned by 3-(aminopropyl)triethoxy silane (APTES), followed by reaction with glutaraldehyde and coupling with avidin. The avidin–biotin technology has been employed to immobilize liposomes in gel beads in LC[22] and in fused silica capillaries in CE using small and large unilamellar liposomes composed of egg phosphatidylcholine and phosphatidylethanolamine with and without different amounts of phosphatidylserine.[23,24] The avidin–biotin binding provides high repeatability of analysis on coated capillaries, but it suffers from quite complicated and time-consuming preparation.[19] An additional disadvantage is the need for using biotinylated lipids, which are expensive and change the lipid characteristics. Further, the presence of a bulky avidin–biotin complex creates a possible place for interaction with drug which can interfere with the drug–lipid interaction.

In this work we aimed at direct covalent binding of liposomes onto the capillary wall. This approach is an alternative to coupling using tresyl, consisting of a four-step reaction that needs two days for preparation.[25] We present here a fast (8 hours) three-step method that enables fine-tuning of the lipid composition and the possible use of lipids extracted from biological matrices. We optimized the coating conditions and measured the coverage of the wall by liposomes using quartz crystal microbalance (QCM) technique. On the optimal liposome coating a set of structurally diverse compounds were separated. The interactions and affinity of the drugs with the liposomes were quantitatively evaluated by retention factors and distribution constants, allowing for comparison of analyte–liposome interactions in liposome dispersions of various types and concentrations.

Materials and methods

Materials

1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-didodecanoyl-*sn-glycero*-3-phosphate (DLPA) and 1,2-didodecanoyl-*sn-glycero*-3-phosphoethanolamine (DLPE) were purchased from Genzyme Pharmaceuticals (Liestal, Switzerland), and 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phospho-L-serine (POPS) from Avanti Lipids (Alabaster, AL, USA). Glutaraldehyde was purchased from Oriola Oy (Espoo, Finland). 3-(Aminopropyl)triethoxy silane, nitric acid, and all investigated drugs were from Sigma (St Louis, MO, USA). Sulfuric acid and HPLC grade methanol were from Mallinckrodt Baker (Deventer, The Netherlands). DMSO and sodium hydroxide (1.0 M) from Oy FF Chemicals Ab (Haukipudas, Finland). Sodium dihydrogen phosphate and pH solutions (7 and 10) used for calibrating the pH meter were purchased from Merck (Darmstadt, Germany). Chloroform from Rathburn (Walkerburn, UK) and acetone and acetic acid from VWR Prolab (Helsinki, Finland). Distilled water was further purified with a Millipore water-purification system (Millipore, Molsheim, France).

Buffer and sample preparation

Sodium acetate and sodium phosphate buffers were prepared by mixing acetic acid and sodium dihydrogen phosphate with 0.1 M and 1 M sodium hydroxide to yield pH 5.5 for sodium acetate buffer and 7.4 or 8.0 for sodium phosphate buffer. The ionic strength of phosphate buffer at pH 7.4 was 20 mM and this buffer was used for all electrophoretic runs and as liposome and analyte solvent. A sodium phosphate buffer at pH 8.0 with an ionic strength of 200 mM was used for treating the capillary wall. Sodium acetate buffer at pH 5.5 ($I = 20$ mM) was used to check the EOF in APTES-coated capillaries. Before use, the buffer solutions were filtered through 0.45 μm syringe filters (Gelman Sciences, Ann Arbor, MI, USA).

The APTES solution for coating was prepared at different concentrations (4%, 8%, and 10% (v/v)) in acetone, acetate at pH 5.5 or phosphate buffer at pH 7.4, or in water. The 2.5% glutaraldehyde solution in phosphate at pH 8 was prepared from a 25% commercial solution.

The samples for CEC were prepared from stock solutions (1 mg mL^{-1} in MeOH). The concentrations of the analytes in the samples for injection were 60–120 μg mL^{-1}, diluted in sodium phosphate buffer at pH 7.4 (ionic strength of 20 mM). DMSO (0.1 or 0.05% in water) or MeOH (15% in background electrolyte solution) was used as a neutral EOF marker. All buffer and sample solutions were stored in a refrigerator.

Preparation of liposomes

Phospholipid vesicles were prepared from POPC, POPS, DLPA, and DLPE lipids. The corresponding transition temperatures are −2, 14, 29, and 31 °C (http://avantilipids.com). POPC (20 mM) and POPS (13 mM) in chloroform, and DLPA (9 mM) and DLPE (10 mM) in chloroform/methanol solution (9/1, v/v) were stored in a freezer. Appropriate amounts of the lipid stock solutions were mixed to obtain the desired compositions. The resulting mixture was evaporated to dryness under a stream of pressurized air, and traces of solvent were removed by evacuation under reduced pressure (8–100 mbar) for at least 16 h. The lipid residues were hydrated in sodium phosphate at pH 7.4 ($I = 20$ mM) at 60 °C for 60 min in a shaking water bath to yield multilamellar vesicles of desired lipid concentration. The vesicle-containing dispersion was vortexed 4 times during the hydration process. The resulting dispersion was processed to large unilamellar vesicles by extrusion 19 times through Millipore

(Bedford, MA, USA) 100 nm pore size polycarbonate filters using a Liposo-Fast extruder and a pneumatic actuator with an external pressure of 3.5 bar (Avestin, Ottawa, Canada). Since extrusion should be carried out well above the transition temperature of the lipids, the DLPA and DLPE dispersions were extruded with a preheated extrusion device, whereas all other dispersions were extruded at RT.

Coating procedure

The attachment of phospholipids to the fused silica capillary was carried out using a three-step coating procedure. The reaction product of aminopropylsilylation of silanol groups on the fused silica surface with glutaraldehyde is an imidoaldehyde, which will react with the primary amino groups of phospholipids. The final optimized coating procedure is schematically presented in Fig. 1.

Aminopropylsilylation of the fused silica capillary

New capillaries were treated in the following way: rinsing (*i.e.* applying a pressure of 940 mbar) for 15 min with 1 M sodium hydroxide or nitric acid, 15 min with water, 5 or 15 min with acetone, 5 or 15 min with air, and rinsing for 5 or 30 min with 4, 8, or 10% APTES dissolved in acetone, water, or acetate buffer at pH 5.5 ($I = 20$ mM), using 50 mbar or 940 mbar pressure at 25 °C or 37 °C. The capillary with tightly closed ends was kept for 2 or 24 hours at 60 °C or overnight at RT and then rinsed with acetone for 10 min and with water for 5 min. Sodium phosphate at pH 7.4 or sodium acetate buffer at pH 5.5 ($I = 20$ mM) was rinsed through the capillary for 30 or 60 min, then −30 kV voltage was applied for 15 min with sodium acetate buffer in the inlet and outlet vials. The EOF was measured using acetate buffer at pH 5.5 and 0.1 or 0.05% DMSO as a neutral marker.

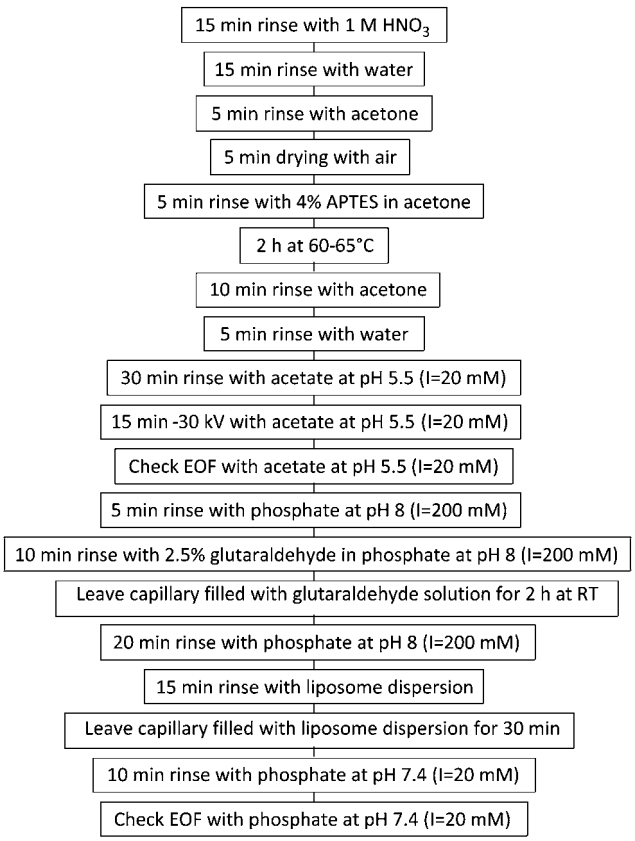

Fig. 1 Liposome coating procedure for fused silica capillaries.

Formation of an iminoaldehyde surface

The aminopropylsilylated capillary was rinsed with sodium phosphate buffer at pH 8.0 for 5 min, then with 2.5% glutaraldehyde solution for 10 min. The capillary was left in the glutaraldehyde solution for 2 h or overnight, resulting in an iminoaldehyde surface.

Covalent coating with phospholipids

The iminoaldehyde coated capillary was rinsed with sodium phosphate buffer at pH 8.0 ($I = 200$ mM) for 20 min, which was followed by rinsing for 15 min with liposome dispersions specified in Table 1. The liposome coating was stabilized by leaving the capillary filled with liposome dispersion at room temperature for 30 min or 4.5 h. The capillary was rinsed with sodium phosphate buffer at pH 7.4 and the EOF was determined.

Capillary electromigration experiments

The CE and CEC experiments were carried out using a Hewlett Packard ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a diode array detector (wavelength 200 nm). Uncoated fused-silica capillaries of 50 μm I.D. (375 μm O.D.) and 30/38.5 cm (length to the detector/total length) were from Polymicro Technologies (Phoenix, AZ, USA). CEC separation conditions were as follows: voltage 25 kV; temperature of the samples and the capillary cassette 25 °C or 37 °C; sample injection 10 s at 10 mbar. The capillary was conditioned shortly before injection by flushing for 2 min with background electrolyte (BGE) solution (sodium phosphate buffer at pH 7.4 with an ionic strength of 20 mM).

Calculation of retention factors

The retention factors, k, were calculated to describe the interactions between the analytes and the phospholipid coating. Retention factors are widely used for quantification of interactions, even though they are highly dependent on the phase ratio (the volume ratio between liposome and aqueous phase), and hence restricted to a specific composition of the BGE solution. The retention factor k was calculated according to eqn (1) from the CEC and CZE data,

Table 1 Liposome dispersions in phosphate buffer pH 7.4 at ionic strength 20 mM used for coating of fused silica capillaries

Concentration/mM	Liposome	mol%
3.0	POPC	100
3.0	POPC/DLPA	80/20
3.0/2.0/1.0	POPC/POPS	80/20
3.0/2.0/1.0	POPC/POPS/DLPA	80/5/15
3.0/2.0/1.0	POPC/POPS/DLPA	80/2.5/17.5
3.0/2.0/1.0	POPC/DLPE/DLPA	75/5/20
3.0/2.0/1.0	POPC/DLPE/DLPA	70/10/20

$$k_{CEC} = \frac{t_m(1 + k_e) - t_{eo}}{t_{eo}} \quad (1)$$

where t_m is the migration time of the analyte in the CEC mode, t_{eo} is the migration time of the EOF marker in the coated capillary, and k_e is the ratio of the migration times of the analyte and the EOF marker under CZE conditions (uncoated capillary).

Calculation of distribution constants

The apparent distribution constant, K_D, is the equilibrium constant for the distribution of an analyte between two phases, and it is equal to the ratio of the molar concentration of the analyte in the stationary phase to the molar concentration of the analyte in the mobile phase. The distribution constant quantifying the interaction between the drugs and the liposomes studied was calculated according to eqn (2) and (3),

$$K_D = \frac{k}{\Phi} \quad (2)$$

$$\Phi = \frac{V_{lip}}{V_{aq}} \quad (3)$$

where k is the retention factor, V_{lip} is the volume of the lipid phase (excluding the internal water), V_{aq} is the volume of the aqueous phase and Φ is the phase ratio.

The calculation of distribution constant considers the chromatographic phase ratio (Φ = volume of the lipid phase/volume of the aqueous phase) and K_D is thus suitable for comparison of analyte–liposome interactions in systems with various concentrations of liposome dispersions. The volume of the lipid phase needed for the phase ratio determination can be calculated utilizing the mass distribution of liposome per area determined by QCM. Considering the inner surface of the capillary and the ratio between the liposome internal water volume and the volume of the liposomal shell (using the known bilayer thickness of 5 nm[10] and density of the dry lipid, 0.95 g mL^{-1} [10]), the volume of lipid phase can be calculated. The resulting phase ratios were in order of 10^{-4}.

Quartz crystal microbalance experiments

The QCM measurements were performed by using the impedance based QCM-Z500 instrument (KSV, Biolin Scientific Oy, Finland). The QCM-Z500 instrument allows for a simultaneous measurement of resonance frequency change (Δf) and quality of the resonance (Q) at six harmonics (fundamental frequency 15, 25, 35, 45 and 55 MHz, corresponding to the overtones, $n = 3$, 5, 7, 9, and 11, respectively) of a 5 MHz crystal. The quality factor (Q) is related to dissipation (D) through $D = 1/Q$. Dissipation change is a measure for distinguishing if adsorbed adlayers on the quartz crystal sensor surface are rigid or soft in their nature. If the adsorbed mass on the crystal is rigid the mass can be determined by the Sauerbrey relation,[26] whereas if it is soft the adsorbed mass and the viscoelastic properties of the adlayer can be determined by using an equivalent circuit modeling.[27] The properties of a selected number of liposomes adsorbed on glutaraldehyde functionalized quartz crystals were analyzed according to the procedure we have presented earlier for non-specifically adsorbed liposomes on silica surfaces.[10] Briefly, the modeled thickness obtained from the equivalent circuit modeling for the adsorbed liposomes reflects the area ratio (AR) between the complete area of the liposome bilayer and the projected area that the liposome occupies on the silica surface. The diameter and height of the actual adsorbed liposomes can then be calculated by utilizing the knowledge of the total area of liposome bilayer (determined by their particle size in bulk) and simple geometrical calculations. The diameter and height of the adsorbed liposomes determined from the above modeling are then used to calculate the total volume of the lipid phase.

For the QCM measurements the silica coated QCM crystals were cleaned by two cycles of exposure to piranha solution (of 1 : 3 hydrogen peroxide : sulfuric acid solution) for 5 to 15 min, then rinsed with distilled water, and dried with nitrogen. The crystals were coated using the same method as for CE capillaries. The crystals were left in the glutaraldehyde solution at RT overnight. For QCM measurements the measurement chamber was filled with phospholipid dispersion (flow velocity of 25 μL min^{-1} for 10 min). After that the flow was stopped for 30 minutes and finally the measurement chamber was rinsed with phosphate buffer at pH 7.4 to remove excess phospholipids (flow velocity of 250 μL min^{-1}).

Results and discussion

Analyte–lipid membrane interactions have been studied by open tubular capillary electrochromatography (OT-CEC) during the last 10 years, *e.g.*, [12,28,29] and papers therein. These studies have mainly been carried out using dynamic lipid coatings that have demonstrated some stability limitations. In this study we developed a method for covalent binding of lipids on fused silica capillaries for the high-throughput screening of drugs (drug–lipid membrane interactions). The fused silica capillary was aminopropylsilylated with 3-(aminopropyl)triethoxy silane (APTES), followed by reaction with glutaraldehyde giving an imidoaldehyde, which then was allowed to react with the primary amino groups of phospholipids. Glutaraldehyde is commonly used in protein crosslinking.[30] Aminopropylsilylation with APTES and the proceeding reaction with glutaraldehyde was carefully optimized (amount of reagent, type of solvent, pH, ionic strength, reaction time, and temperature). The following step in the reaction involved covalent binding of the free aldehyde group in glutaraldehyde to phospholipids containing primary amino groups. There is one reference from 1969 discussing the reaction of glutaraldehyde with tissue lipids, strengthening our hypothesis of the procedure.[31] The phospholipids investigated in this work are shown in Fig. 2. The study involved phospholipids with both primary and tertiary amino groups, and the aim was to verify the effect of primary amino groups in phospholipids for successful covalent binding. The reaction scheme of the covalent binding process is illustrated in Fig. 3.

The coating procedure was optimized in order to reach stable and high coverage phospholipid binding. All steps (APTES, glutaraldehyde, and phospholipid) were optimized separately.

Reaction with APTES and glutaraldehyde

High aminopropylsilylation coverage was needed on the silica surface for the proceeding reactions (see reaction scheme in Fig. 3). Accordingly, we were aiming at stable and high

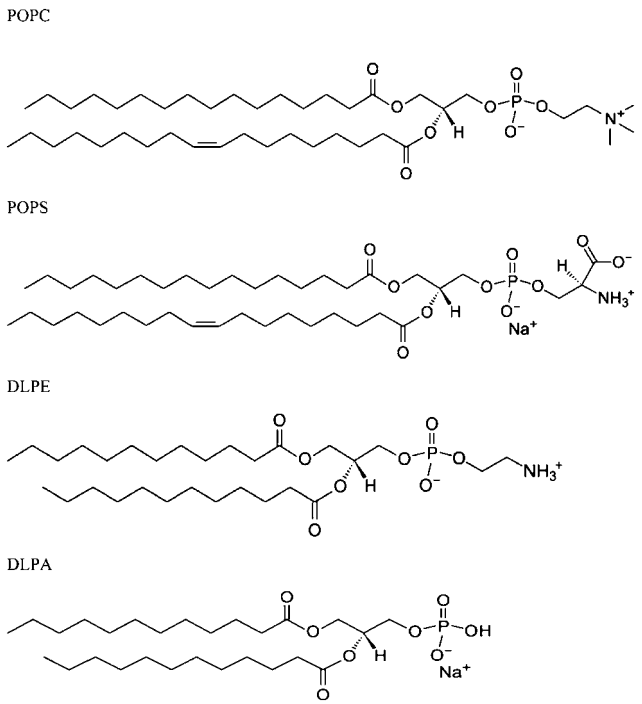

Fig. 2 Structures of phospholipids investigated.

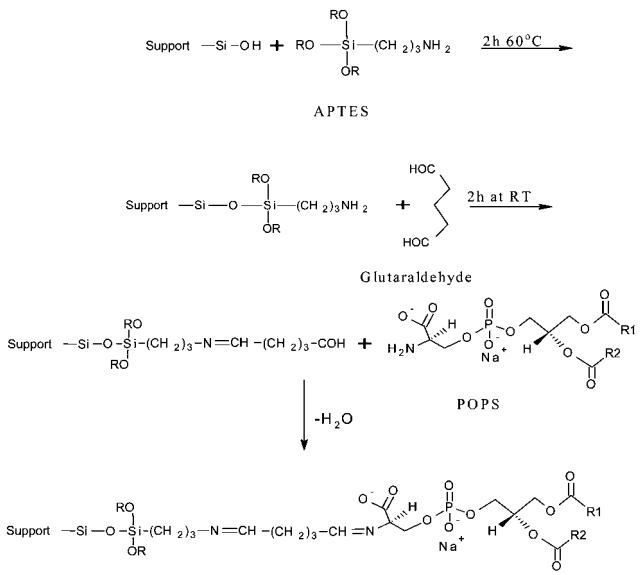

Fig. 3 Reaction scheme on the covalent binding of phospholipids on fused silica capillaries.

electroosmotic flows in the aminopropylsilylated capillary. Optimization parameters for aminopropylsilylation included the type of prerinsing solution (sodium hydroxide, nitric acid), concentration of APTES (4%, 8%, or 10%, v/v), APTES solvent (acetone, water, or phosphate buffer), the length of APTES treatment (2 h and 12 h), and the buffer for EOF determinations (acetate buffer pH 5.5 and phosphate at pH 5.5 and 7.4, all with an ionic strength of 20 mM).

First, the effect of preconditioning before reaction with APTES was investigated using sodium hydroxide and nitric acid. The highest EOF values were obtained using nitric acid in the preconditioning step (Fig. 4). In these tests the APTES concentration was 4% in acetone and there was a 2 h waiting step at ambient temperature before the EOF measurement. Acetate buffer at pH 5.5 (ionic strength of 20 mM) was used as BGE solution. The other APTES concentrations (8% and 10%) investigated did not increase the EOF, proving that the fused silica capillary was sufficiently aminopropylsilylated using 4% APTES solution. Longer APTES treatment times could not be used due to blockage of the capillary. The effect of pH (acetate at 5.5 and phosphate buffer 7.4 with an ionic strength of 20 mM) on the EOF was investigated, and because acetate buffers resulted in more repeatable EOF values, the pH 5.5 buffer was selected for further EOF measurements.

Glutaraldehyde was dissolved in phosphate buffer of 200 mM ionic strength at pH 8.0, because under these conditions only a little reversibility in the reaction of glutaraldehyde with primary amino group has been observed.[32] The concentration of the glutaraldehyde solution adopted from the literature[30] was equal to 2.5%. No significant difference in the EOF values was observed when the capillary was left filled with glutaraldehyde solution for 2 h or overnight. Glutaraldehyde diminished the positive charges of the aminopropylsilylated surface, resulting in very low EOF values (Fig. 4).

Covalent binding of phospholipids

The glutaraldehyde activated capillary was subsequently coated with different types and concentrations of liposomes (in the form of large unilamellar vesicles). In the reaction of glutaraldehyde with a nucleophilic primary amino group an exceptionally stable

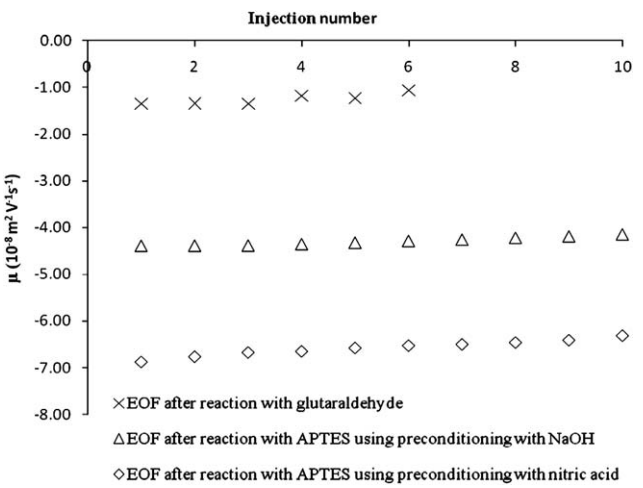

Fig. 4 Electroosmotic flow of capillaries after aminopropylsilylation with 4% APTES in acetone using preconditioning with 1 M sodium hydroxide (triangles) or 1 M nitric acid (square/rhombus). The crosses show the EOF after reaction with glutaraldehyde. Running conditions: capillary 30/38.5 cm (ID/OD; 50/360 μm); voltage −20 kV; temperature of the capillary cassette 25 °C; sample injection for 10 s at 10 mbar; UV-detection at 214 nm. Before each injection the capillary was rinsed for 2 min with the given BGE solution; after reaction with glutaraldehyde rinsing with phosphate buffer at pH 7.4, ionic strength of 20 mM, and after reaction with APTES rinsing with acetate buffer pH 5.5, ionic strength of 20 mM. The EOF marker was 0.05% DMSO in water.

linkage should be formed.[30] ^{1}H NMR and mass spectrometric analyses have been employed to prove the covalent bonding between aldehyde and amino group of phosphatidylethanolamine and phosphatidylserine.[33] However, glutaraldehyde reacts not only with primary amino groups but also with thiol, phenol, and imidazole groups of amino acids and proteins.[34] The reactivity of different aldehydes decreases in the following reactivity order: ε-amino, α-amino, guanidinyl, secondary amino, and hydroxyl groups.[30]

In order to test the need for primary amino groups for reaction between phospholipids and the iminoaldehyde surface, various types of liposomes were designed with a broad range of compositions and concentrations (Fig. 2 and Table 1). Two of the selected phospholipids contained primary amino groups (POPS and DLPE), the third was without any amino group (DLPA), and the fourth contained a tertiary amino group (POPC).

The increase in the time of the liposome treatment from 30 min up to 4.5 h had almost no effect on the retention of some neutral model steroids (see Fig. 5). Longer liposome treatment times improved the peak shapes of the analytes and slightly increased the EOF, suggesting better liposome coverage of the capillary. However, because the difference was very small the shorter liposome treatment time of 30 min was selected for further experiments to limit the analysis time.

As expected, the composition of the liposomes clearly affected the EOF mobilities (Fig. 6). The capillary wall was the most negatively charged using 80/20 mol% POPC/POPS liposomes of 3 mM. Considering the reaction scheme (Fig. 3) it is clear that a sufficient amount of (imino)aldehyde groups must be available for the reaction with the primary amino groups of the phospholipids to achieve high liposome coverage of the capillary surface. The effect of aminopropylsilylation of fused silica capillaries is shown in Fig. 7B. Some of the capillaries exhibited very low EOF values after aminopropylsilylation, which resulted in low coverage of liposomes, as seen in the low retention factors of the analytes (see Fig. 7B). Thus, the EOF value measured after the aminopropylsilylation of the fused silica capillary with APTES should be equal to or higher than -5×10^{-8} m^2 V^{-1} s^{-1} to provide high liposome coverage of the capillary.

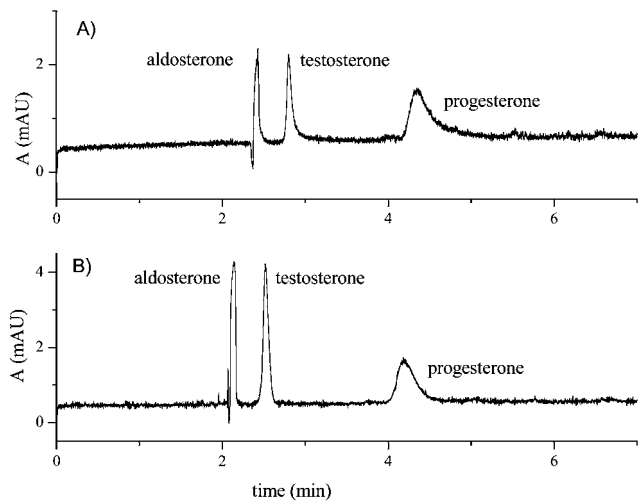

Fig. 5 Effect of liposome treatment time on the separation of neutral steroids. 3 mM 80/5/15 mol% POPC/POPS/DLPA dispersion in phosphate buffer of pH 7.4 and ionic strength of 20 mM was left in the capillary for 30 min in (A) and for 4.5 h in (B). Separation conditions were as follows: capillary 30/38.5 cm (ID/OD; 50/360 μm); separation voltage 25 kV; temperature of the capillary cassette 25 °C; sample injection for 10 s at 10 mbar; UV-detection at 245 nm. Before each injection the capillary was rinsed for 2 min with the BGE solution (phosphate buffer at pH 7.4, ionic strength of 20 mM). The EOF marker was 15% methanol in BGE.

Covalent *versus* dynamic coating

The stability of the liposome coating was tested to see the effect of the phospholipid primary amino group on the covalent reaction of phospholipids with iminoaldehyde (see the reaction scheme in Fig. 3). The EOF of 3 mM 80/20 mol% POPC/POPS was stable up to 80 injections, opposite to 3 mM 80/20 mol% POPC/DLPA that fluctuated and resulted in run-to-run decreased EOF values (Fig. 8). In the latter liposome dispersion both phospholipids lack primary amino groups, which clearly affects the reaction with iminoaldehyde. However, despite some fluctuations, the liposomes lacking primary amino groups were bound to the iminoaldehyde layer to some extent (evidenced from the surprisingly good coating obtained with POPC/DLPA). The tertiary amino group in PC is most probably poorly reactive but the hydroxy group in PA may be involved in the reaction. Another aspect to consider is the transition temperature of the lipids. The experiments in Fig. 7 were carried out at ambient temperature, and keeping in mind that the transition temperature of DLPA is 31 °C, the POPC/DLPA lipid layer may be slightly stiffer than the POPC/POPS bilayer. We have observed that phospholipid coatings will be more stable on capillary surfaces while in the rigid gel state,[13] however, in this case both liposomes were clearly in the liquid fluid state during capillary electrophoretic experiments.

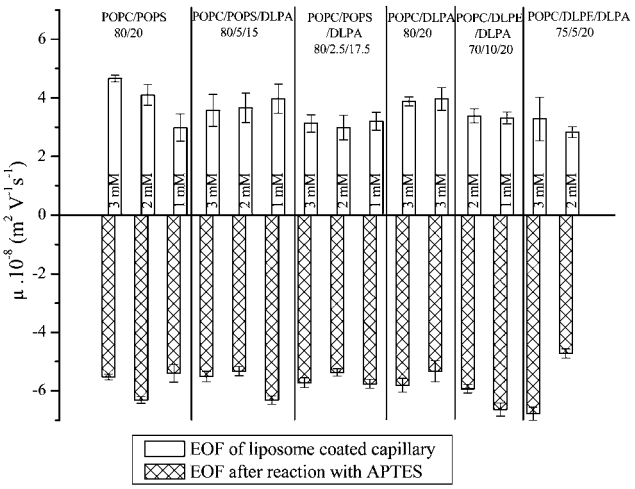

Fig. 6 EOF values in capillaries after reaction with APTES and liposomes. CEC separation conditions: capillary 30/38.5 cm (ID/OD; 50/360 μm); separation voltage 25 kV; temperature of the capillary cassette 25 °C; sample injection for 10 s at 10 mbar; UV-detection at 214 nm. Before each injection the capillary was rinsed for 2 min with the BGE solution (phosphate buffer at pH 7.4, ionic strength of 20 mM). The EOF marker was 0.05% DMSO in water.

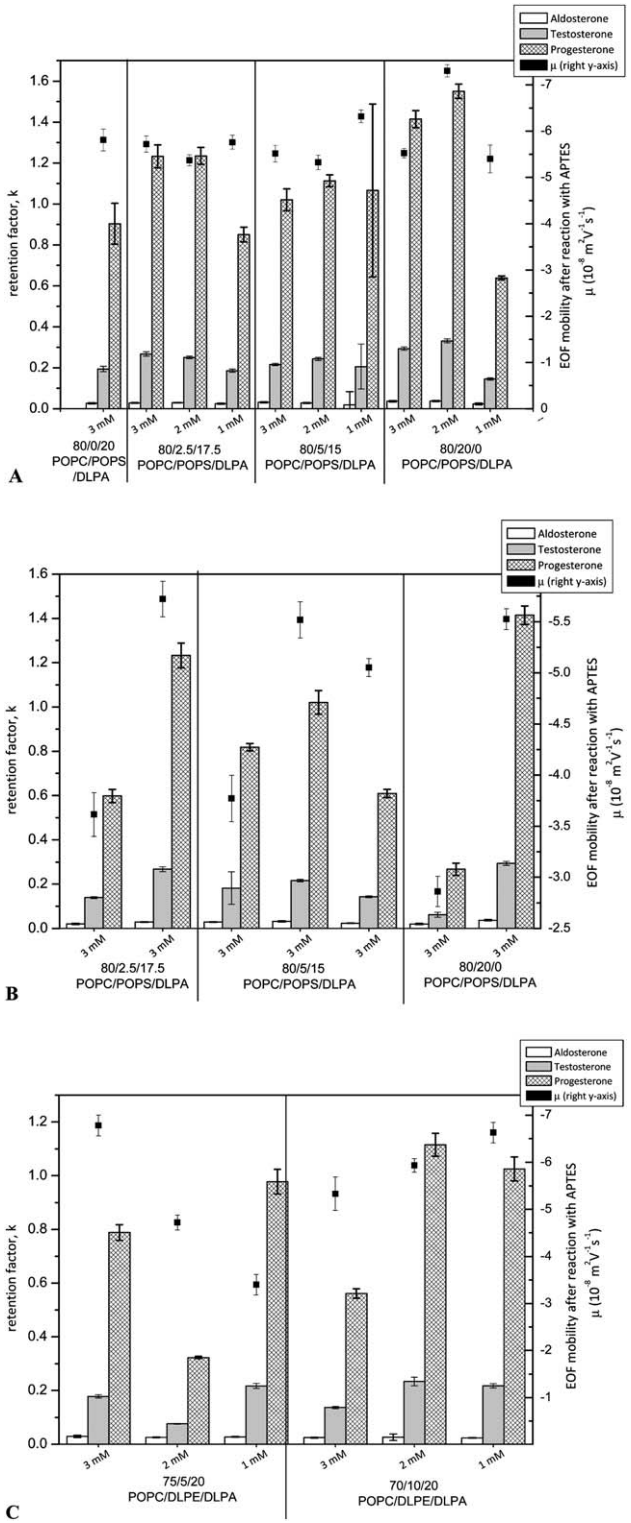

Fig. 7 Retention factor for steroids using different liposome coatings and EOF values after reaction with APTES. (A) PC/PS/PA coatings; (B) reproducibility data for PC/PS/PA coatings; and (C) PC/PE/PA coatings. CEC separation conditions were as follows: capillary 30/38.5 cm (ID/OD; 50/360 μm); separation voltage 25 kV; temperature of the capillary cassette 25 °C in (A) and (B) and 37 °C (C); sample injection for 10 s at 10 mbar; UV-detection at 214 nm (EOF-marker) and 245 nm (steroids). Before each injection the capillary was rinsed for 2 min with phosphate buffer at pH 7.4, ionic strength of 20 mM. The EOF marker was 0.05% DMSO in water.

Retention factors

The lipophilicity of the various liposome surfaces was compared by determining the retention factors of the neutral steroids aldosterone, testosterone, and progesterone (Fig. 7A). The highest retention factors of all steroids were found for coatings made from 2 or 3 mM liposome dispersions. Stable covalent coatings were obtained even with liposomes containing only 2.5 mol% of POPS (possessing primary amino group). The lower retention factors obtained with the 3 mM 80/20 mol% POPC/DLPA dispersion suggest that the phospholipids in this coating were bound only dynamically to the iminoaldehyde surface. There were some variations in the EOF values after aminopropylsilylation, but generally good covalent coatings were obtained when the EOF was around -5×10^{-8} m^2 V^{-1} s^{-1}, or even more positively charged. Comparing results on successful and unsuccessful liposome coatings, the role of good aminopropylsilylation is obvious. This is exemplified in Fig. 7B for 3 mM liposome dispersions with increasing molar concentration of POPS (2.5, 5, and 20 mol%). Lower retention factors were obtained for all model analytes when the fused silica capillary was only partly aminopropylsilylated by APTES treatment, however, the effect was pronounced for the most hydrophobic steroid (progesterone). The electropherograms showing the effect of successful aminopropylsilylation on the separation of three neutral steroids are seen in Fig. 9.

Liposome coating and the electrophoretic runs for studying lipid membrane–analyte interactions with the POPC/DLPE/DLPA coatings were carried out at 37 °C. The higher running temperature was selected to ensure that all single lipids were in the liquid fluid state. Biological membranes generally exist in the liquid crystal phase *in vivo*, and this phase is probably the most relevant for the majority of processes taken place in biological membranes. Phosphatidylethanolamine has a primary amino group and should hence have strong interactions with aldehyde groups. However, the retention factors of the steroids on the capillary coated with POPC/DLPE/DLPA (Fig. 7C) were lower

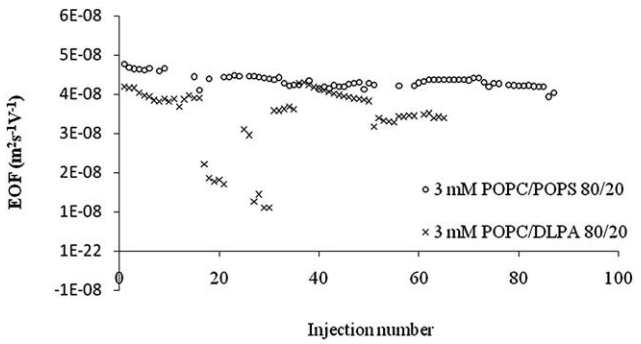

Fig. 8 Comparison between dynamic (POPC/DLPA) and covalent (POPC/POPS) phospholipid/liposome coatings. The liposomes were dissolved in phosphate buffer at pH 7.4, ionic strength of 20 mM. CEC separation conditions were as follows: capillary 30/38.5 cm (ID/OD; 50/360 μm); separation voltage 25 kV; temperature of the capillary cassette 25 °C; sample injection for 10 s at 10 mbar; UV-detection at 214 nm. Before each injection the capillary was rinsed for 2 min with the BGE solution (phosphate buffer at pH 7.4, ionic strength of 20 mM). The EOF marker was 15% methanol in BGE.

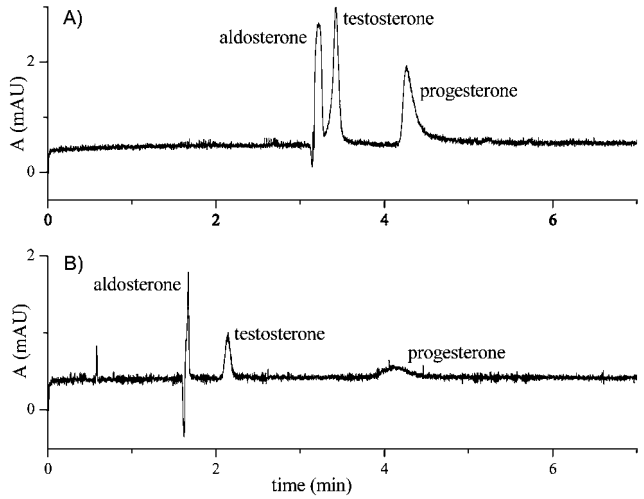

Fig. 9 Effect of successful aminopropylsilylation on the separation of three neutral steroids. The EOF after reaction with APTES was -2.86×10^{-8} m^2 s^{-1} V^{-1} in (A) and -5.53×10^{-8} m^2 s^{-1} V^{-1} in (B). The capillary was coated with a 3 mM 80/20 mol% POPC/POPS liposome dispersion in phosphate buffer at pH 7.4 and ionic strength of 20 mM. EOF marker was 15% methanol in phosphate buffer and running conditions as in Fig. 5A.

than those containing similar molar percentage of POPS (Fig. 7A). Here a direct comparison of the retention factors should not be made because even though the molar fractions of POPS and DLPE were the same, the molar fractions of the other two lipids were different and the working temperature was higher in Fig. 7C than in Fig. 7A.

In our previous open-tubular CEC study on dynamically coated phospholipid capillaries we tested the retention of steroids using various phospholipid compositions.[18] In that work the tested steroids had stronger interactions with PS-containing membranes than with PA-containing membranes. The retention factor of the most hydrophobic steroid progesterone on the capillary coated with 3 mM PC/PS (80/20 mol%) lipid solution was 0.3 using dynamically attached phospholipids[18] and 1.4 using covalently bound liposomes (this work). Since the interaction of progesterone with the liposomes should be the same, the difference in retention factors suggests higher coverage of the capillary wall with liposomes when using the covalently bound liposomes.

Determination of distribution constants by CE and QCM

Drug lipophilicity is an important characteristic in numerous pharmaceutical applications. The frequently used log *P* value is based on the distribution of the drug between *n*-octanol and water and it fails in mimicking the biomembrane environment, because it considers only non-polar interactions[1,2] (and citations herein). There is a need for rapid techniques yielding information on drug lipophilicities. A covalently attached phospholipid phase seems to be an excellent alternative, since it enables tuning the specific membrane composition and gives results in short time (especially when compared to time consuming membrane dialysis experiments that are much used in pharmaceutical industry).

A model set of drugs were selected and their retention factors on the 3 mM 80/20 mol% POPC/POPS liposome coated capillaries were determined (Table 2). In order to convert the retention factors into distribution constants, the phase ratio (see eqn (2)) needed to be calculated. Determination of the phase ratio is not straightforward and approximations are typically done. Usually only retention factors, that are strongly dependent on the concentration or amount of the immobilized phase, are stated. In this work we measured the phase ratio using data obtained by a quartz crystal microbalance. QCM is a highly efficient technique for determining adsorbed masses of substances on a solid support.[27,35] We have previously investigated the deposition of various liposomes on silica-coated quartz crystals and observed that depending on the lipid composition and the surrounding solvent, the liposomes will either form supported bilayers or remain on the surface as truncated vesicles.[10] Fig. 10 shows the normalized frequency change for a selected number of liposome compositions. The large frequency and dissipation (see Table 3) shifts indicate that the liposomes adsorb on the iminoaldehyde-functionalized silica surface as complete liposomes. This is also supported by the fact that the normalized frequency shifts for the measured overtones do not superimpose with each other (not shown), and that the ratio between liposome diameter and height is close to 1 (Table 3). There is a clear dependency on the liposome composition and the final mass adsorbed. The final mass for the adsorbed liposomes increased in the following order 3 mM POPC/DLPA < 3 mM POPC/POPS < 2 mM POPC/POPS. This is in line with the retention factors measured for phospholipid coated capillaries with corresponding liposome compositions (see Fig. 7A). The higher amount of adsorbed liposomes for 2 mM POPC/POPS compared to 3 mM POPC/POPS is caused by the difference in the degree of aminopropylsilylation of the silica surface. The degree of aminopropylsilylation of the silica surface has a clear effect on the EOF mobility (Fig. 7B), but it also influences the final mass of adsorbed liposomes which is then reflected in the retention factors (Fig. 7).

Table 2 Retention factors, log *P* values and distribution constants of tested compounds using the capillary coated with 3 mM 80/20 mol% POPC/POPS dissolved in phosphate buffer of pH 7.4 and ionic strength of 20 mM

Analyte (charge at pH 7.4)	3 mM 80/20 mol% POPC/POPS			
	k_{CEC}	log *P*	log K_D	K_D
Benzthiazide (+)	2.140	2.575	3.657	4540.9
Bupivacaine (+)	0.370	3.312	2.898	791.2
Ketanest (+)	0.190	3.012	2.605	402.8
Lidocaine (+)	0.120	2.196	2.392	246.8
Prilocaine (+)	0.180	2.029	2.586	385.1
Sotalol (+)	0.200	0.24	2.628	424.7
Tramadol (+)	0.280	2.316	2.771	590.8
4-Pregnen-11β, 17α-diol-3,20-dione (0)	0.090	2.735	2.296	197.9
Aldosterone (0)	0.040	0.706	1.890	77.7
Androsterone (0)	0.160	3.932	2.543	348.9
Corticosterone (0)	0.170	1.952	2.568	369.9
Dexamethasone (0)	0.290	2.033	2.795	623.1
DHEA (0)	0.690	3.305	3.162	1451.3
Progesterone (0)	1.410	3.827	3.476	2995.3
Testosterone (0)	0.290	3.179	2.794	622.0
Hydrochlorothiazide (−)	0.010	−0.021	1.170	14.8

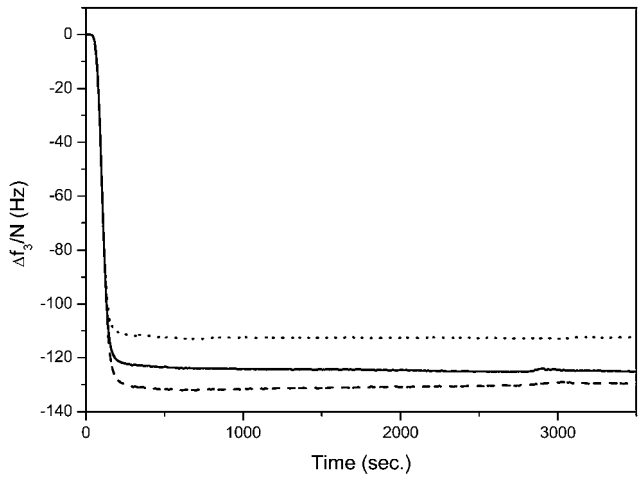

Fig. 10 Normalized frequency shift during liposome adsorption to a glutaraldehyde functionalized silica quartz crystal. 3 mM POPC/POPS (solid line), 2 mM (dashed line) and 3 mM POPC/DLPA (dotted line).

Table 3 Data on liposome coatings by QCM and there from derived liposome volumes and masses

Composition of liposomes	PC/PS 3 mM	PC/PS 2 mM	PC : PA 3 mM
Modeled thickness/nm	24.8 (0.2)	26.2 (0.4)	21.9 (0.1)
Mass distribution of liposome per area/μg cm^{-2}	2.48	2.62	2.19
$(\Delta f_3/N)$/Hz	124.6 (0.3)	131.1 (0.4)	112.6 (0.1)
ΔD_3 (10×10^{-6})	11.0 (0.3)	12.0 (0.2)	9.3 (0.2)
d_{liposome}	116.03	112.88	165.71
h_{liposome}	114.86	119.7	141.28
d/h	1.01	0.94	1.17
Volume of water phase/m^3	7.56×10^{-10}	7.56×10^{-10}	7.56×10^{-10}
Mass of liposome/g	1.50×10^{-6}	1.58×10^{-6}	1.32×10^{-6}
Mass of lipids/g	3.39×10^{-7}	3.56×10^{-7}	2.30×10^{-7}
Volume of lipid/m^3	3.57×10^{-13}	3.75×10^{-13}	2.42×10^{-13}
Phase ratio	4.72×10^{-4}	4.96×10^{-4}	3.21×10^{-4}

The thickness of the liposome layer, liposome diameter, and liposome height obtained from QCM analysis were used to calculate the mass distribution of liposome per area, the mass of lipid and accordingly, the volume of the lipid phases used in CEC (density approximations were done). The results are shown in Table 3. The phase ratios are in the order of 10^{-4}, similar as in liposome electrokinetic chromatography.[36] The distribution constants of anaesthetic drugs (bupivacaine, prilocaine, lidocaine) and POPC/POPS liposomes are very similar to the distribution constants with negatively charged POPC/POPG liposome, that were determined using electrokinetic capillary chromatography.[36] The dynamically coated capillaries exhibited lower coverage of the silica surface when compared to the covalently attached ones. The modeled thickness was at least five times lower using dynamically coated capillaries.[37] Employing only physical adsorption, the lipid bilayers were forming patches because of poorer adhesion to the silica surface originating from repulsive interactions between the liposomes and the silica surface.[16]

The correlation between distribution constants and log P values for charged and neutral analytes is shown in Fig. 11. Even

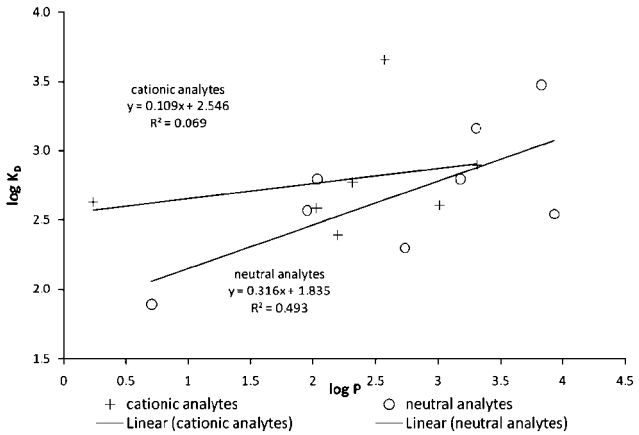

Fig. 11 Correlation between log K_D and log P. log P values are derived from Advanced Chemistry Development Software V8.14 for Solaris. Experimental conditions for calculation of K_D values are as in Fig. 8.

though the neutral analytes exhibited higher correlation when compared to the charged ones, the correlation is still weak (correlation coefficient of 0.49). As expected, the drug partitioning into octanol only describes hydrophobic interactions and excludes other possible interactions, *i.e.* van der Waals interactions and hydrogen bonding, between the drugs and the liposomes.

Conclusions

A new covalent coating method was developed for the preparation of liposome immobilized capillaries. The effectiveness of the coating was demonstrated by separation of a set of structurally diverse analytes. The capillaries exhibited good repeatability and reproducibility, long lifetime and satisfactory time of preparation (about 8 hours). Liposomes composed of only 2.5% of POPS (a phospholipid containing a primary amino group) were covalently bound to the iminoaldehyde-coated capillary. Using QCM we observed that the immobilized lipid film consists of a layer of practically non-truncated liposome vesicles, which did not collapse into a lipid bilayer. Phase ratios of the immobilized lipid and aqueous volume in the coated capillary were calculated from the lipid mass, determined by QCM. A model set of diverse drugs were applied into the capillary with an immobilized liposome layer of 80/20 mol% POPC/POPS. The retention factors k were measured and considering the amount of liposomes immobilized on the silica wall, the distribution constants were calculated. With such an approach it is possible to fine-tune the liposome composition or to use lipids extracted from biological matrixes in order to have a wide range of phospholipid-coated capillaries, applicable to drug–membrane interaction studies.

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