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Preparation approaches of the coated capillaries with liposomes in capillary electrophoresis

Jie Mei [a], Yan-Ping Tian [a], Wen He [b], Yu-Xiu Xiao [a,*], Juan Wei [a], Yu-Qi Feng [c]

[a] College of Pharmacy, Wuhan University, Wuhan 430071, China
[b] Renmin Hospital, Wuhan University, Wuhan 430060, China
[c] Department of Chemistry, Wuhan University, Wuhan 430072, China

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ABSTRACT

The use of liposomes as coating materials in capillary electrophoresis has recently emerged as an important and popular research area. There are three preparation methods that are commonly used for coating capillaries with liposomes, namely physical adsorption, avidin–biotin binding and covalent coupling. Herein, the three different coating methods were compared, and the liposome-coated capillaries prepared by these methods were evaluated by studying systematically their EOF characterization and performance (repeatability, reproducibility and lifetime). The amount of immobilized phospholipids and the interactions between liposome or phospholipid membrane and neutral compounds for the liposome-coated capillaries prepared by these methods were also investigated in detail. Finally, the merits and disadvantages for each coating method were reviewed.

1. Introduction

Utilization of liposomes in capillary electrophoresis (CE) has received great attention in the past several years [1–3]. While liposomes can be used as pseudostationary phases in CE, their usage as coating materials has attracted wider attention, because liposomes do not affect the properties of background electrolyte (BGE) solution and very small amounts of liposomes are required for coating. Liposome-coated capillaries have been applied for the separation of proteins and peptides [4–10], inorganic anions [11], steroids [12–14], phenols [13], chiral compounds [15–17], and amino acids [18]. Furthermore and importantly, the liposome-coated capillaries have recently been used to study biomembrane properties and interactions because of their structural similarity to biomembrane lipid bilayers [19–24].

There are a variety of techniques by which phospholipids or liposomes can be immobilized on the inner wall of capillaries. These techniques can be broadly categorized as: physical adsorption, avidin–biotin binding and covalent coupling.

Of these three methods, the physical adsorption is the most widely used technique to prepare the liposome-coated capillaries. In 2002, Cunliffe et al. first reported a simple physical adsorption method [4], in which capillaries were coated with 1,2-dilauroyl-*sn*-phosphatidylcholine by repeatedly rinsing with a solution of small unilamellar vesicles. This physical adsorption methodology was further developed by Riekkola and her co-workers [5,6,12–18,21,22]. The behavior and effectiveness of the physically adsorbed coating are influenced by various factors, including the ionic strength and type of buffer, the presence of calcium ion, the size and lamellarity of vesicles, the type and concentration of phospholipids, etc. Surfactant molecules also have various effects on the structure and properties of lipid bilayers or lipid vesicles [6,11]. For examples, when surfactants and phospholipids were mixed in different ratios, the EOF of coated capillaries varied from fully reversed to near zero [11]. To improve the stability of this physically adsorbed coating, oligomerized phospholipids instead of phospholipids were used as coating materials [7,25]. In 2002, Örnskov et al. reported an attractive approach to immobilize intact liposomes in capillaries based on electrostatic interaction [19]. In their pioneering work, the inner wall of capillaries was coated with derivatized agarose, which contains a positively charged quaternary ammonium moiety. Subsequently, the negatively charged liposomes were immobilized on the polymer coating via electrostatic interaction. This reported procedure generated stable coatings under neutral pH and ionic strength up to 20 mM. The intra- and inter-capillary variations in EOF were *ca.* 4% RSD ($n = 45$) and *ca.* 10% RSD ($n = 5$), respectively.

Avidin contains four identical subunits which can bind to biotin with a high degree of affinity and specificity. The avidin–biotin binding technique has been widely used to achieve highly yielded stable liposome-containing LC stationary phases [26] and Yang

* Corresponding author at: College of Pharmacy, Wuhan University, Wuhan 430071, China. Tel.: +86 27 68759990; fax: +86 27 68759850.
E-mail address: yuxiuxiao@yahoo.com.cn (Y.-X. Xiao).

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et al. first applied this method to CE in 1998 [27]. In Yang's pioneering work, biotinylated liposomes, composed of egg phosphatidylcholine (PC) and biotinylated phosphatidylethanolamine, were successfully immobilized in the capillaries in the presence of avidin.

Phospholipids or liposomes can also be covalently bound to the inner wall of capillaries if appropriate ligands are introduced inside the capillaries. In 2008, our group reported a new covalent coupling method for coating capillaries with liposome solutions [24]. Our three-step method includes silanization, activation and liposome coupling.

Due to our interests in the applications of liposome-coated capillaries to studying interactions, especially quantitative interactions, between biomembrane and compounds by CE, herein in this paper, we compared systematically the three preparation approaches (physical adsorption, avidin–biotin binding and covalent coupling) of liposome-coated capillaries in CE. The EOF characterization and the performance (repeatability, reproducibility and lifetime) of the liposome-coated capillaries prepared by the three coating methods were studied in detail. We have also investigated the amount of immobilized phospholipids and the applications in quantitative study of compound–biomembrane interactions for the liposome-coated capillaries prepared by the three different coating methods.

2. Experimental

2.1. Chemicals and reagents

All the chemicals in the experiments were of analytical grade and purchased from National Medicine Co. Ltd. (Shanghai, China) unless noted otherwise. Soybean PC (>94%) and dimyristoylphosphatidylethanolamine (DMPE) (>99%) were purchased from Lipoid Co. (Ludwigshafen, RP, Germany). 1,2-Dioleoylphosphatidylethanolamine-*N*-(cap biotinyl) (biotin-PE) was from Avanti Polar Lipids (Alabaster, AL, USA). Egg-white avidin (M_r 66,000) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) were bought from Sigma–Aldrich (St. Louis, MO, USA). HEPES and Tris were from Amresco (Solon, OH, USA). γ-Glycidoxypropyltrimethoxysilane (GPTMS) (>97%) was obtained from Alfa Aesar (Ward Hill, MA, USA) and 3-aminopropyltriethoxysilane (APS) (>95%) was from the Chemical Plant of Wuhan University (Wuhan, Hubei, China). The model steroids used in this study were obtained as follows: androstenedione (Huayi Co. Ltd., Shanghai, China), hydrocortisone (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), progesterone and testosterone (Sanjing Chemical Pharmaceutical Co. Ltd., Wuhan, China). Ultrapure distilled water with resistance greater than 17 MΩ was obtained from SG ultrapure water treatment equipment (German).

2.2. BGE and sample preparation

The concentration of HEPES buffer was 25 mM at pH 7.4 adjusted with 1.0 M sodium hydroxide. This solution was used as the liposome solvent and BGE unless noted otherwise. The stock solutions of steroids were prepared as 2 mg/mL in methanol. The concentrations of steroids for injection were 50 μg/mL (progesterone), 20 μg/mL (androstenedione), 20 μg/mL (hydrocortisone) and 20 μg/mL (testosterone) in BGE. DMSO (0.1%, v/v) in water was used as the marker of EOF.

2.3. Liposome preparation

Liposomes were prepared as described in our early publication [24]. PC/DMPE mixtures were used in the physical adsorption and covalent coupling methods, while PC/biotin-PE mixtures were used in the avidin–biotin binding method. The phospholipid composition of liposomes was expressed as mole percentage in the mixtures. An appropriate amount of phospholipids was dissolved in chloroform/methanol (2:1, v/v) in a round-bottom flask. The round-bottom flask was placed in a rotary evaporator at 35 °C to produce a thin layer of phospholipids on the inner surface of the flask. Trace amounts of any remaining solvent were removed under vacuum overnight. By vigorous vortex, the lipid film was hydrated in 25 mM HEPES buffer (pH 7.4) to yield a dispersion of multilamellar vesicles (MLVs) with a lipid concentration of *ca.* 5 mM. The MLVs were then processed to large unilamellar vesicles by frozen–thawed-extrusion method. The dispersion of MLVs was frozen at −20 °C for 30 min and thawed at 45 °C for 15 min, and the suspension was shaken between each cycle. After five frozen/thawed cycles, the obtained suspension was extruded 15 times through 0.1 μm pore size polycarbonate filters using an EmulsiFlex-C5 extruder (Avestin, Canada). The prepared liposome suspension was stored at 4 °C in the dark.

2.4. Liposome characterization

The average diameter of prepared liposomes was determined by dynamic light scattering (DLS) (3000 HS Zetasizer, Malvern Co., U.K.) at 25 °C and at an angle of 90°. To get an appropriate concentration, the liposome suspension was diluted with HEPES buffer prior to the measurements. The zeta potential of liposomes was determined by laser doppler velocimetry (LDV) (3000 HS Zetasizer, Malvern Co., U.K.) at 25 °C. Three DLS and LDV measurements were carried out for each sample.

2.5. Capillary coating

In order to get an objective assessment, the pre-conditioning step of capillaries, the type and concentration of phospholipids, liposome preparation method, buffer solutions and buffer pH value were kept the same as much as possible in three different preparation methods. Pretreatment of a fresh fused-silica capillary (50 μm I.D., 375 μm O.D. and 2 m length) was as follows: the capillary was rinsed with 1 M NaOH for 2 h, water for 1.5 h, 1 M HCl for 2 h, water for 1 h, and methanol for 30 min; and dried under nitrogen flow at 160 °C for 3 h.

2.5.1. Physical adsorption method

The physical adsorption method employed an optimized procedure described in the literature [12]. After pre-conditioning, the capillary was rinsed with BGE for 10 min and with a liposome solution for 10 min at 995–998 mbar. The resulting capillary was then allowed to stand still with the liposome solution filled for 15 min and subsequently washed with BGE for 5 min to remove any unbound liposome.

2.5.2. Covalent coupling method

The covalent coupling method for coating of capillaries with liposomes has been developed in our research group and details could be found in our earlier publication [24]. Briefly, the method includes three steps. The first step was epoxy-diol coating, in which a solution of 20% (v/v) GPTMS in dry toluene was pumped through a pretreated capillary. After the solution stayed in the capillary at 110 °C for 5 h, the capillary was rinsed with toluene and water. Subsequently, 0.1 M HCl was flushed through the capillary. After several minutes, the flow of 0.1 M HCl was stopped, and the capillary was left to sit overnight at room temperature, with the ends capped. The second step was activation with tresyl chloride. The epoxy-diol-coated capillary was washed with acetone–water (9:1, v/v) and dry acetone in sequence. Then a mixed solution of 17 mL tresyl chloride in dry acetone and 34 mL pyridine was pumped

through the capillary. After 35 min at room temperature, the capillary was rinsed with acetone and then flushed with nitrogen. The final step was liposome coupling, in which the activated capillary was rinsed with BGE for 15 min and the liposome solutions for 20 min, followed by staying still at 25 °C for 3 h, with ends capped. After this treatment, the capillary was washed with BGE for 30 min to remove the unbound liposomes.

2.5.3. Avidin–biotin binding method

The avidin–biotin binding method was optimized from a reported procedure [27]. First of all, a pre-conditioned capillary was treated with APS [10% (v/v) in water] for 5 min and was placed in an oven at 95 °C for 30 min. This procedure was repeated twice. The capillary filled with APS solution was left still overnight, and was rinsed with 50 mM phosphate (pH 7.0) for 15 min. Subsequently, a glutaraldehyde solution (2%, v/v) in 50 mM phosphate (pH 7.0) was passed through the amino-fused silica capillary for 30 min to activate it, followed by rinsing with 50 mM phosphate (pH 7.0) for 10 min. Next, avidin (1 mg/mL) in 50 mM phosphate (pH 7.0) was continuously drawn into the aldehyde-activated capillary by vacuum for 30 min, and the capillary was incubated overnight at room temperature by capping the ends. The resulting avidin-coupled capillary was then rinsed with Tris–HCl buffer (0.5 M, pH 7.5) to remove the unreacted aldehyde residue, followed by rinsing with BGE. Finally, the biotinylated liposome solution was drawn through the capillary and allowed to stay in the capillary for 30 min, followed by rinsing with BGE.

2.6. Determination of the amount of phospholipids immobilized on the coated capillaries

In order to determine the amount of phospholipids immobilized on the capillary wall, a 2 m long coated capillary was used, and the immobilized phospholipids were completely eluted with a solution of chloroform/methanol (2:1, v/v) and collected. The amount of phospholipids in the collected eluent was determined by using Bartlett's method [28]. Briefly, the collected eluent was first evaporated to dryness at 80 °C under vacuum, and 1 mL water and 0.5 mL strong sulfuric acid were then added to dissolve the residue. The obtained solution was heated in an oven of 170 °C for 3 h, and then 0.1 mL of 30% (v/v) hydrogen peroxide was added. The solution was returned to the oven for 1.5 h more to complete the combustion and to decompose all the peroxide. After this, 40 μL of 5% ammonium molybdate and 40 μL of the Fiske–SubbaRow reagent were added. The solution was diluted to 1 mL with water, mixed thoroughly, and heated for 7 min at 100 °C. The absorbance (A) at 830 nm of the final solution was recorded. The amount of phospholipids immobilized on the coated capillary was calculated according to the phosphorus standard curve, which was established in our experiments and as follows:

$$A = -0.015 + 24.6C \quad (r = 0.9988,\ n = 5)$$

where C is the concentration of standard phosphorus (potassium dihydrogen phosphate) solution, expressed in unit of μmol/mL. Linear range is 0.005–0.04 μmol/mL.

2.7. CE conditions

The CE experiments were carried out with a CAPEL-105 CE system (LUMEX, Russia) which consisted of a high voltage power supply, a multi-wavelength UV–VIS detector, a water-cooling circulating device for temperature control of the cartridge containing a capillary, and 1.5 X Chrom&Spec software providing data acquisition and evaluation. CE was carried out under the following conditions unless noted otherwise: fused-silica capillaries (50 μm I.D. and 375 μm O.D.) in 40 cm total length with 31.5 cm to detector; 25 mM HEPES buffer (pH 7.4) being used as BGE and filtered through a 0.22 mm cellulose acetate filter; voltage 15 kV, providing a current of ~5 μA; a capillary cassette temperature of 25 °C; UV detection at 214 nm; injection of EOF marker or samples at 30 mbar for 5 s. Between runs, the capillaries were rinsed with BGE for 2 min.

3. Results and discussion

3.1. Liposome preparation and characterization

Our previous work [24] has shown that the liposome preparation method was a key factor for the size distribution of liposomes. We finally chose a frozen–thawed-extrusion method to prepare liposomes, due to the fact that the polydispersity of the liposomes prepared by this method was much narrower than that by an extrusion method where the frozen–thawed step was omitted [24].

The size and charge of liposomes influence not only the final formation and stability of the coating [10,12,13,22,29,30] but also the electrophoretic behaviors of analytes on coated capillaries [13,22]. Average diameters of liposomes determined in this study were approximately 120 ± 2 nm (n = 3) for PC/DMPE (95%/5%) and 124 ± 3 nm (n = 3) for PC/biotin-PE (95%/5%), respectively. The size similarity between PC/DMPE and PC/biotin-PE liposomes suggested that the composition of liposomes had little influences on their size. However, the composition of liposomes had significant impacts on their zeta potentials. The PC/DMPE (95%/5%) liposomes had a zeta potential of −7.6 ± 0.3 mV (n = 3), indicating that PC/DMPE (95%/5%) liposomes had almost neutral surfaces. On the other hand, PC/biotin-PE (95%/5%) liposomes had a lower zeta potential (−38.0 ± 0.4 mV, n = 3) and carried negative charges on their surfaces since biotin-PE consisted of anionic phospholipids (Fig. 1).

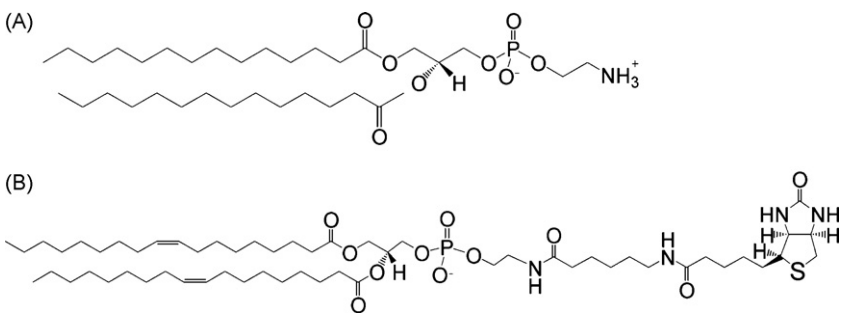

Fig. 1. Structures of DMPE (A) and biotin-PE (B).

3.2. Optimization of the coating conditions for the avidin–biotin binding method

Prior to make an objective assessment, the coating procedure for the three methods used in this study have been optimized. For the physical adsorption method, Riekkola and co-workers have done intensive work on the optimization of coating conditions, including liposome rinsing time, the waiting time for the final coating, buffer solutions, buffer pH, etc., and they used the EOF and the separation of neutral steroids to evaluate coating quality [12]. We utilized their best coating conditions in our standard physical adsorption method, as shown in Section 2.5.1. For the covalent coupling method, the coating conditions have been systematically studied and optimized in our earlier study [24], and our optimal procedure was shown in Section 2.5.2.

Yang et al. [27] have developed an avidin–biotin binding method to immobilize liposomes on the capillary wall. Unfortunately, few details were reported with regards to the optimization of coating conditions and the performance of the coated capillaries. Their procedure for the immobilization of biotinylated liposomes on the avidin-bound capillary included two steps: first, a biotinylated liposome solution without avidin was drawn through the avidin-bound capillary for 30 min followed by rinsing with BGE; second, a biotinylated liposome solution with a low concentration of avidin was immediately drawn into the capillary and retained for 15 min, followed by flushing with avidin solution for 5 min and incubating for 15 min. Several liposome layers were assembled on the capillary's inner wall because of the second step. Initially, we applied the same procedure to prepare the avidin–biotin coating capillaries and the EOF was found to be remarkably suppressed. The migration time of DMSO was approximately 53 min in a 40 cm (effective length 30.5 cm) × 50 μm I.D. coated capillary, which is not ideal for analyzing neutral compounds. In order to decrease the amount of immobilized liposomes and thus analysis time, in our experiments, the second step was omitted as described in Section 2.5.3. In that case, only one liposome layer was theoretically formed on the inner wall of the capillary. As expected, there was a clear enhancement on the EOF and the DMSO migration time was about 6.4 min.

To get better coating performance, three parameters in this avidin–biotin binding method have been further optimized: concentration of the glutaraldehyde solution (0.25, 0.5, 1, 2, 5%, v/v), rinsing time of the glutaraldehyde solution (0.5, 0.75, 1, 1.5, 2 h), and rising time of the liposome solution (10, 20, 30, 45, 60 min). The EOF and the separation of neutral steroids were used to evaluate the coating quality in the optimization process. The results showed that 2% glutaraldehyde concentration, rinsing the glutaraldehyde solution for 30 min, and rinsing the liposome solution for 30 min appeared to be sufficient (data not shown). Furthermore, a rinsing step with 50 mM phosphate (pH 7.0) for 15 min after the silanization of capillaries with APS was added to the coating procedure. This extra step can clearly improve the stability of coating.

3.3. Comparison of three preparation methods for the coated capillaries

As shown in Section 2.5, the physical adsorption method was very simple, fast (only 40 min), so easy to optimize coating procedure and conditions, and provided a good flexibility regarding the composition of liposomes and the renewal of the coating. Another obvious feature for this method was that liposomes directly interacted with capillary inner wall during coating and hence there was no any spacer group between the physical adsorption coating and capillary inner wall. Additionally, the physical adsorption method was environmentally friendly.

In contrast, both the covalent coupling method and the avidin–biotin coupling method were complicated and time-consuming (more than 24 h), so not easy to optimize coating procedure and conditions, and did not provide a good flexibility regarding the composition of liposomes and the renewal of the coating. Moreover, several spacer groups were produced between the phospholipid coating and capillary inner wall because of GPTMS silanization and tresyl chloride activation for the covalent coupling method, and APS silanization, glutaraldehyde activation and avidin-coupling for the avidin–biotin coupling method, respectively. In addition, the covalent coupling method needed a lot of organic reagents and so was environmentally unfriendly; while the avidin–biotin coupling method was very expensive and changed the liposome characteristics due to the utilization of biotinylated lipid.

3.4. The amount of phospholipids immobilized on the coated capillaries by the three methods

The coated capillaries were prepared by the three methods with PC/DMPE (95%/5%) or PC/biotin-PE (95%/5%) liposomes as the coating materials, and the amount of phospholipids immobilized on the capillaries was determined as described in Section 2.6. The experiments showed that phospholipids were successfully immobilized on the inner wall of the coated capillaries prepared by the three methods. The amount of phospholipids immobilized on the capillary coated by physical adsorption (10.24 nmol/m) was slightly lower than those by covalent coupling (12.09 nmol/m) and by avidin–biotin binding (11.58 nmol/m).

Interestingly, the amount of immobilized phospholipids that we measured on the avidin–biotin binding capillary was inconsistent with the number reported by Yang et al. [27]. In their work, it was postulated that the intact spherical liposomes were coated on the capillary wall and about 15 liposome layers were likely to form on the inner surface of the capillary, based on liposome characteristics and the amount of immobilized phospholipids (47 nmol/m). Therefore, the immobilization amount should be *ca.* 3 nmol/m if the capillary is coated by only one liposome layer. As mentioned in Section 3.2, by using our avidin–biotin binding method, only one liposome (or phospholipid) layer was formed on the capillary's inner wall. However, we obtained an immobilization amount of 11.58 nmol/m for our coating method, about four times higher than the number determined by Yang et al. [27]. This inconsistency suggests that the final coating form should be phospholipid bilayers and/or deformed vesicular layers rather than intact spherical liposome layers in the avidin–biotin binding capillaries.

3.5. EOF characterization of the coated capillaries by the three methods

It is well known that the EOF is dependent on the zeta potential of a capillary's inner surface. In a coated capillary, EOF is related to the surface charge of coating materials because the zeta potential is dictated mainly by the surface charge of coating materials [31].

In our research, the effect of BGE's pH on the EOF mobility of liposome-coated capillaries has been investigated. In order to maintain a high buffer capacity, a phosphate (20 mM)–citric acid (10 mM) mixed buffer was used as BGE, whose pH varied from 3.4 to 8.0. The EOF mobility at each pH level was the average of four repetitious experiments. The EOF of an uncoated capillary was also determined as a reference by employing the same BGE. The results from PC/DMPE (95%/5%) liposome coating and PC/biotin-PE (95%/5%) liposome coating were shown in Fig. 2.

For an uncoated capillary, the ionization of silanols was low and the change in EOF was insignificant if the buffer pH varied below 5.5. When buffer pH was higher than 5.5, the variation of pH had significant impacts on the EOF, because when buffer pH increased, the silanols were gradually deprotonated, which con-

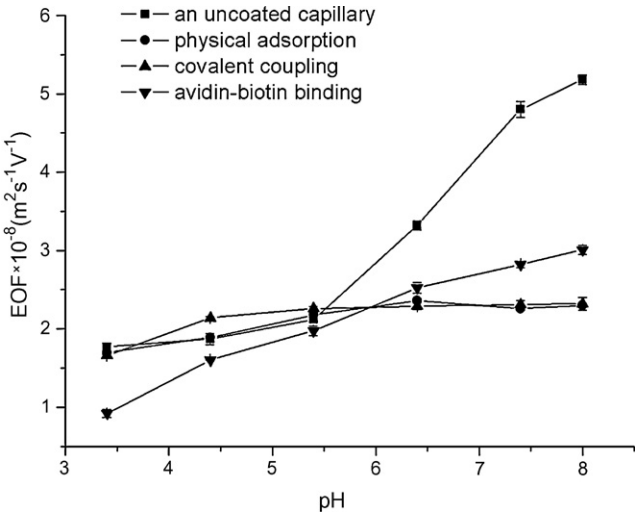

Fig. 2. The plots of EOF versus pH of BGE for an uncoated capillary, coated capillaries prepared by physical adsorption, covalent coupling and avidin–biotin binding, respectively. Liposomes used for coating contained 95:5% PC/DMPE or 95:5% PC/biotin-PE. CE conditions: 31.5/40 cm capillaries (31.5 cm to detector, I.D.: 50 μm, O.D.: 375 μm); UV detection at 214 nm; 15 kV; injection at 30 mbar for 5 s; temperature at 25 °C; phosphate (20 mM)–citric acid (10 mM) mixed buffer as BGE. DMSO (0.1%, v/v) in water was used as EOF marker.

sequently increased the zeta potential and the EOF. For PC/DMPE (95%/5%) liposome-coated capillaries prepared by physical adsorption or covalent coupling, their EOF mobility remained very similar under a pH range of 3.4–8.0. This is because the intrinsic p*K*a is 0.8 for the phosphate group of PC, while p*K*a for the phosphate and amino group resided in DMPE is 0.5 and 9.6, respectively [32,33]. The charges of both PC and DMPE are independent of the buffer pH under our investigated pH range. However, for PC/biotin-PE (95%/5%) liposome-coated capillaries prepared by avidin–biotin binding, their EOF mobility increased when buffer pH increased, probably because of the ionizable functionalities presented in biotin-PE.

As indicated in Fig. 2, the EOF was suppressed for all the coated capillaries compared to the EOF for the uncoated capillary when the buffer pH was higher than 5.5. For physical adsorption coating, it is probably because the negatively charged silanols were partially shielded by uncharged phospholipids (at pH 5.5–8.0), leading to the suppressed EOF. Therefore, the EOF suppression under high pH conditions would reflect the quality of phospholipid (or liposome) coating on the capillary wall, and the change in EOF was frequently utilized as an important indicator to evaluate the immobilization of phospholipids (or liposomes) on the inner wall of capillaries [4–7,10–15,18,20–22]. For example, Gulcev and Lucy [10] used the change in EOF as an indicator not only for the formation of the phospholipid (or liposome) coating but also for the completion of the coating.

While the coated phospholipids or liposomes accounted for the EOF suppression for the physical adsorption method, the reasons for the EOF suppression in the covalent coupling method were unclear, because the capillary was treated by the other steps before liposomes were coated via covalent binding. The pretreatment included an epoxy-diol coating and tresyl chloride activation. Our previous work [24] has shown that the EOF mobility in epoxy-diol-coated and tresyl chloride-activated capillaries was significantly lower than that in the uncoated capillary, while similar to that in the liposome-coated capillary. Therefore, for the covalent coating method, one cannot rely on the EOF change to judge the formation or completion of phospholipid (or liposome) coating.

Similarly, the capillary prepared by the avidin–biotin binding method was also modified by coupling steps before the immobilization of biotinylated liposomes, namely APS modification, glutaraldehyde activation and avidin-coupling. Again, the EOF change cannot be used as an indicator to evaluate the presence of a phospholipid or liposome coating. However, avidin, a basic tetrameric glycoprotein with a 10.4 p*K*a value, carried a net positive charge under the conditions (pH 7.4) used in our experiments. Therefore, the avidin-coated capillary had a reversed EOF direction towards the anode, while the EOF was redirected towards the cathode after being flushed with a negatively charged biotinylated liposome solution (data not shown). This redirecting behavior of EOF can clearly indicate the formation of phospholipid or liposome coating.

3.6. The performance of the coated capillaries by the three methods

Repeatability and reproducibility are important factors in the evaluation of capillary performance. Repeatability has been studied through the RSD of the EOF mobilities for 30 runs. The experiments were carried out at pH 7.4 BGE and the results were shown in Table 1. In general, the liposome-coated capillaries prepared by the covalent coupling or avidin–biotin binding method have better repeatability than those prepared by the physical adsorption method. This is apparently due to much stronger interactions between phospholipids and the capillary wall when the covalent coupling or avidin–biotin binding method is utilized.

Our data also indicated the amount of DMPE influenced significantly on the repeatability for the physical adsorption coating, while having little impact for the covalent coupling coating. When a similar physical adsorption method was applied by Hautala et al. to prepare liposome-coated capillaries using 100% POPC, poor repeatability with a 20.7% RSD value for 35 runs was reported [12]. Our experiments showed that the repeatability (5.9% RSD) was dramatically enhanced when 5% DMPE was added. Interestingly, further increasing DMPE amount to 20% resulted in a sharp decrease of the repeatability. The reason for this sharp decrease is not clear to us and further investigation is currently being carried out in our lab.

The reproducibility was obtained from four capillaries in pH 7.4 BGE (5 injections for each capillary), and the RSD values of their EOF mobilities were also summarized in Table 1. Apparently, the avidin–biotin binding and the covalent coupling methods were highly reproducible while the reproducibility from the physical adsorption method was not satisfied. In addition, unlike the repeatability for the physical adsorption coating, the expected positive effect of increasing DMPE amount on the reproducibility was not observed.

Another key property for coated capillaries is lifetime. To evaluate the lifetime of coated capillaries, the separating experiment of neutral compounds was carried out every five days in a one-month period (five or six repetitions for each day), and the RSD of the EOF mobilities was recorded. In addition, the change of the retention factor (k) of neutral compounds was also recorded during the experiment, because it can reflect the variation in the amount of immobilized phospholipids on the capillary wall [34,35]. The retention factor was calculated according to the formula 1, which has been discussed in our early publication [24]. Four steroids (hydrocortisone, androstenedione, testosterone, and progesterone) were selected as model neutral compounds. The coated capillaries (liposomes used for coating contained 95:5% PC/DMPE or 95:5% PC/biotin-PE) were stored with BGE filled at 4 °C and used directly without refreshing with liposome solutions.

$$k = \frac{v^{+}_{\mathrm{EOF}} - v^{+}_{\mathrm{obs}}}{v^{+}_{\mathrm{obs}}} \quad (1)$$

Table 1
Repeatability and reproducibility of the liposome-coated capillaries prepared by physical adsorption, covalent coupling and avidin–biotin binding, respectively.

Coating method	Physical adsorption		Covalent coupling		Avidin–biotin binding
Liposome composition	PC:DMPE (95:5)	PC:DMPE (80:20)	PC:DMPE (95:5)	PC:DMPE (80: 20)	PC:biotin-PE (95:5)
Repeatability RSD % (n = 30)	5.9%	14.9%	4.6%	4.0%	3.1%
Reproducibility RSD % (n = 4)	25.1%	17.0%	7.3%	6.7%	3.2%

where v^{+}_{obs} is apparent velocity of uncharged solutes and v^{+}_{EOF} is the velocity of EOF in a liposome-coated capillary.

A month later, the RSD of the EOF mobility was increased to 6.43% for covalent coupling capillaries and 15.04% for avidin–biotin binding capillaries. As indicated in Fig. 3, a slight decrease of k was observed in both covalent coupling and avidin–biotin binding capillaries, which suggested that small amounts of immobilized phospholipids might leak out for both coated capillaries. One can see that the decreasing rate of k from covalent coupling capillaries was smaller than that from avidin–biotin binding capillaries (Fig. 3). For example, the k value of progesterone decreased from 1.08 to 0.91 (15.7% loss) in a month for covalent coupling capillaries, while from 1.54 to 0.99 (35.7% loss) under the same conditions for avidin–biotin binding capillaries. This indicated that covalent coupling coating had better stability than avidin–biotin binding coating. All four neutral compounds could be completely separated on both covalent coupling and avidin–biotin binding capillaries after they were stored for one month. However, those capillaries prepared by physical adsorption had poor lifetime. After 15 days, the coating was significantly dissolved or drop from the inner wall of capillaries since the RSD value of EOF mobilities was up to 32.6% and the overall resolution of four neutral compounds decreased markedly.

Overall, the covalent coupling and the avidin–biotin binding capillaries have longer lifetime (at least one month) than the physical adsorption capillaries (less than 15 days). The stability of the covalent coupling coating is superior to the avidin–biotin binding coating.

3.7. The suitability of the coated capillaries by the three methods

The liposome-coated capillaries were prepared by three different methods as described in Section 2.5 and were used to separate four neutral steroids. The composition of liposomes is PC/DMPE (95%/5%) or PC/biotin-PE (95%/5%). The neutral steroids can be separated through their hydrophobic interactions with the coating, and thus their retention factors describe the characteristics of the coating in a more straightforward manner [22].

The separation of the four neutral steroids under the same BGE buffer system using coated and uncoated capillaries was outlined in Fig. 4. The uncoated capillary suffered very poor separation of the model neutral steroids, while all coated capillaries achieved great separation. Our data showed that the exact same migration order of the four neutral steroids was followed by using different coated capillaries. In addition, the migration order was correlated with hydrophobic properties of these compounds: the stronger

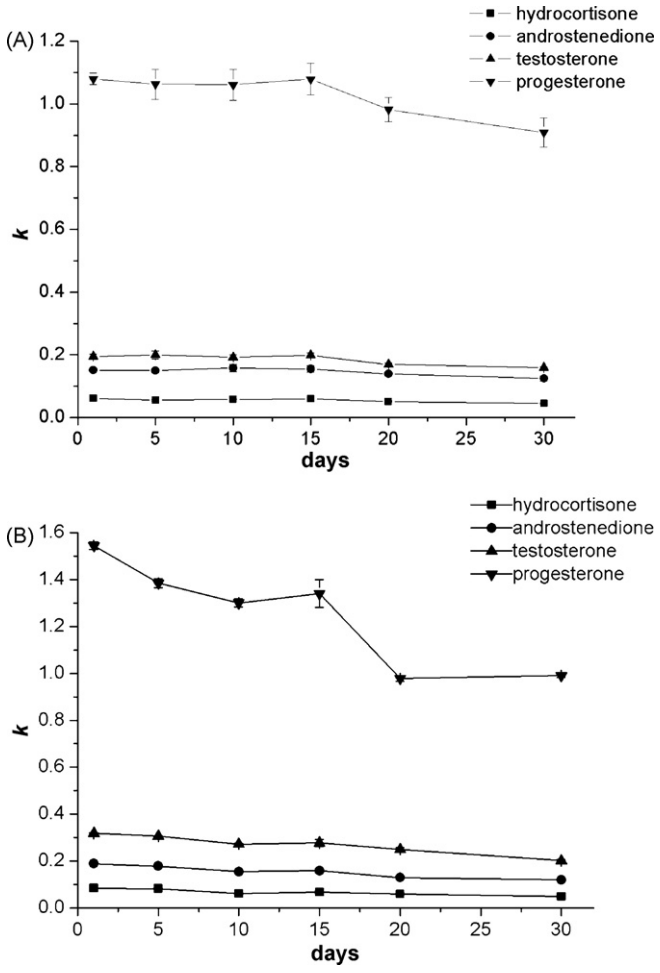

Fig. 3. Stability of the liposome-coated capillaries prepared by covalent coupling (A) and avidin–biotin binding (B). The liposomes used for coating contained 95:5% PC/DMPE or 95:5% PC/biotin-PE. CE conditions: 31.5/40 cm capillaries (31.5 cm to detector, I.D.: 50 μm, O.D.: 375 μm); UV detection at 214 nm; 15 kV; injection at 30 mbar for 5 s; temperature at 25 °C; 25 mM HEPES buffer (pH 7.4) as BGE.

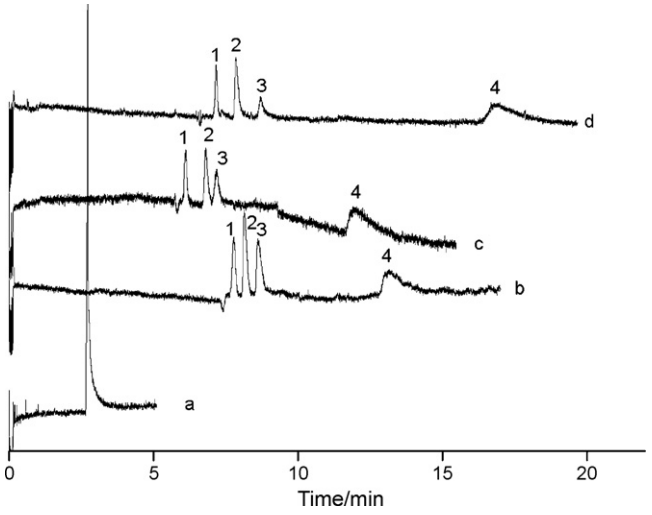

Fig. 4. The separation of four steroids on an uncoated capillary (a), a physical adsorption capillary (b), a covalent coupling capillary (c), and an avidin–biotin binding capillary (d). 1, Hydrocortisone; 2, androstenedione; 3, testosterone; 4, progesterone. CE conditions similar to those in Fig. 3.

Table 2
Log K values determined on a coated capillary prepared by physical adsorption, covalent coupling and avidin–biotin binding, respectively. All data was obtained in one day. The liposomes used for coating contained 95:5% PC/DMPE or 95:5% PC/biotin-PE.

Log K (n = 3)	Physical adsorption	Covalent coupling	Avidin–biotin binding
Hydrocortisone	4.17 ± 0.02	4.20 ± 0.04	4.37 ± 0.001
Androstenedione	4.48 ± 0.01	4.60 ± 0.03	4.71 ± 0.002
Testosterone	4.70 ± 0.004	4.7 ± 0.16	4.94 ± 0.002
Progesterone	5.37 ± 0.003	5.45 ± 0.09	5.63 ± 0.004

hydrophobicity, the longer migration time. Our result is consistent with the findings of Riekkola and co-workers [21].

Normalized retention factor (K) was utilized to quantitatively evaluate the interactions of model compounds with the coating materials. The K value of each steroid was the average for three runs and its calculation was by the formula (2) [24].

$$K = \frac{k}{B} \quad (2)$$

where B is the amount of phospholipids immobilized in the inner wall of an effective length of coated capillaries, expressed in the unit of mol.

Of the three coating methods, the avidin–biotin binding method gave the largest K values and provided the strongest interactions with liposome coating for the neutral steroids, as shown in Table 2. There are several reasons that could account for this. First of all, when the capillary's inner wall was treated by the silanization of APS in the first step of the avidin–biotin binding, its surface hydrophobicity would increase. The following glutaraldehyde activation and avidin-coupling of the aminopropyl-silica capillary would further enhance the interactions. Secondly, the presence of the biotinyl group in biotinylated lipids would change the liposome characters and thereby perhaps increased the compound–lipid membrane interactions. Furthermore, the strong interactions might be related to the large surface area of the avidin–biotin coating.

The covalent coupling coating had medium K values and thus medium interactions with the neutral compounds. Similar to the avidin–biotin binding, the GPTMS silanization and tresyl chloride activation steps allowed the covalent coupling capillaries to gain extra hydrophobic interactions for the neutral compounds. Obviously, the covalent coupling gained less extra interactions than the avidin–biotin binding. The K values from the physical adsorption coating were the smallest ones and could reflect the real interactions between compounds and liposome or phospholipid membrane without extra interferences because there was no any spacer group between the physical adsorption coating and capillary inner wall.

4. Conclusions

Three different methods used to prepare liposome-coated capillaries were compared. It was found that the amount of immobilized phospholipids was almost independent on the preparation methods. The EOF suppression under high pH conditions for the physical adsorption method and the EOF redirecting behavior for the avidin–biotin binding method can clearly indicate the formation of phospholipid or liposome coating in the inner wall of capillaries. However, for the covalent coupling method, the EOF change cannot be used as an indicator to evaluate the presence of phospholipid or liposome coating. The physical adsorption method was very simple, fast, easy and hence provided a good flexibility regarding the composition of liposomes and the renewal of the coating, and additionally suitable to study the real interactions between compounds and liposome or phospholipid membrane without extra interferences; but the coated capillaries prepared by this method suffered poor repeatability, reproducibility and lifetime which would suppress their application in quantitative researches of interactions between compounds and liposome or phospholipid membrane. In contrast, the avidin–biotin binding and the covalent coupling method provided higher repeatability, higher reproducibility and longer lifetime for the coated capillaries, while bearing several disadvantages. Both of them are complicated and time-consuming. Additional disadvantages associated with avidin–biotin coupling are: first, the presence of the biotinyl group in biotinylated lipid changes the liposome characteristics; second, APS silanization, glutaraldehyde activation and avidin-coupling produced extra spacer groups, and thus extra interactions which might interfere with the interactions between analytes and liposome or phospholipid membrane. These problems could be against the wide utilization of avidin–biotin coupling capillaries in CE for the study of the interactions between compounds and liposome or phospholipid membrane. The covalent coupling coating encounters a similar problem: it gained extra interactions with analytes due to GPTMS silanization and tresyl chloride activation, although these extra interactions are weaker than those gained by the avidin–biotin binding method. However, we believe that this problem could be resolved by decreasing the number of spacer groups connecting to silica-supports and liposomes and those studies are being carried out in our group.

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