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Journal of Chromatography B, 841 (2006) 38–51
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Analysis of liposomes by capillary electrophoresis and their use as carrier in electrokinetic chromatography☆

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Received 9 January 2006; accepted 15 March 2006
Available online 6 May 2006

Abstract

This contribution reviews work about liposomes in the context of electrically driven separation methods in the capillary format. The discussion covers four topics. The one broaches the application of liposomes as pseudo-stationary phases or carriers in vesicle or liposome electrokinetic chromatography (EKC) in the way as microemulsions and micelles are used; it includes the chromatographic use of liposomal bilayers as stationary phases attached to the wall for capillary electrochromatography (CEC). The second topic is the characterization and separation of liposomes as analytes by capillary electrophoresis (CE). Then the determination of distribution coefficients and binding constants between liposomes and ligands is discussed, and finally work dealing with peptides and proteins are reviewed with lipid bilayers as constituents of the electrically driven separation system.

Keywords: Liposome; Phospholipids; Capillary electrochromatography; Capillary electrophoresis; Vesicles; Pseudostationary phase; Proteins; Peptides

Contents

1. Introduction

Liposomes are self-assembled vesicles commonly consisting of phospholipid bilayers enclosing an aqueous solution, with other lipid aggregates found as well. These model organelles have been widely used to mimic processes occurring at cell membranes. The main phospholipids of *Eukarya* are phosphatidylcholine (PC, the most common phospholipid in natural membranes), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin (SM); for the abbreviations and symbols of the compounds and for their structures see Table 1. These substances are amphiphilic (they possess a hydrophilic and a lipophilic entity) and many are zwitterionic. Liposomes can be multi- or unilamellar. The diameter of unilamellar vesicles

☆ This paper is part of a special volume entitled "Analysis of proteins, peptides and glycanes by capillary (electromigration) techniques", dedicated to Zdenek Deyl, guest edited by I. Miksik.

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doi:10.1016/j.jchromb.2006.03.031

Table 1
List of compounds for liposome formation including structural formulae and symbols used in the text

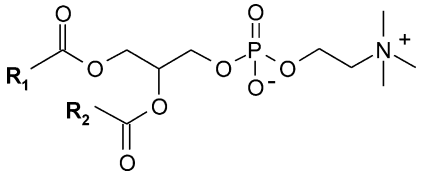
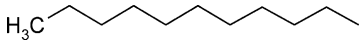
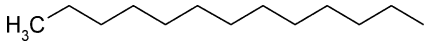
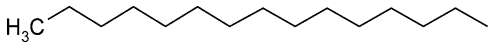
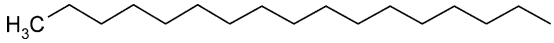
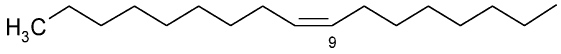
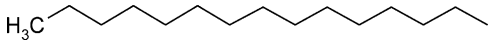
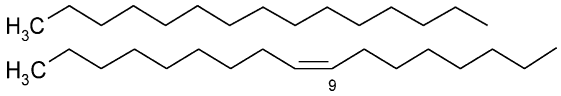
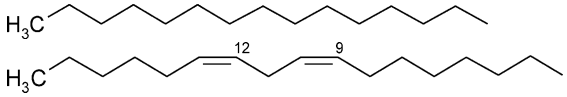
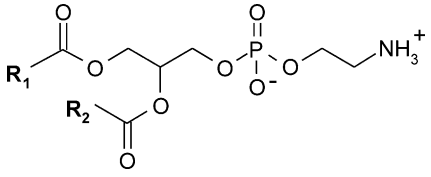
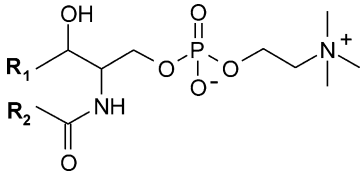
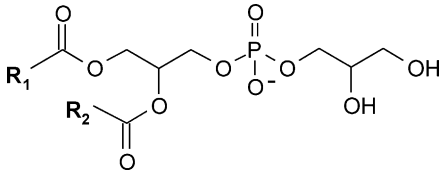
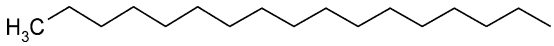
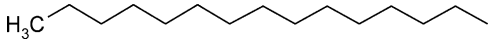
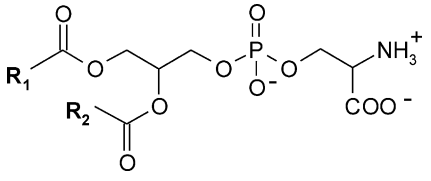
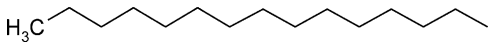
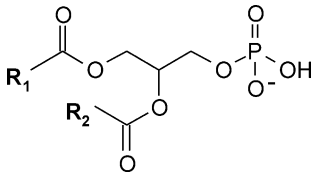
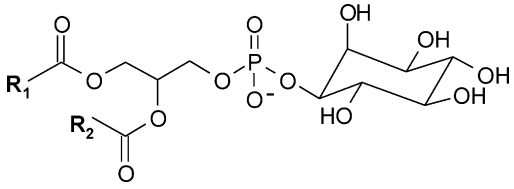
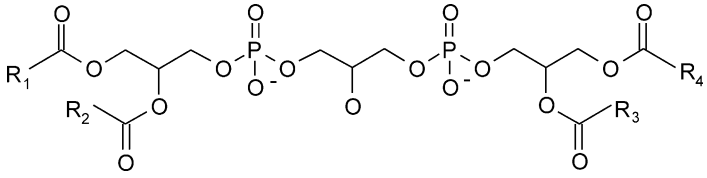
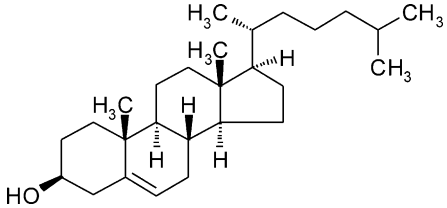
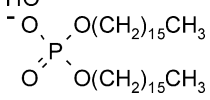
Compound	Symbol	Structure	*R*
Phosphatidylcholine	PC		
Dilauroylphosphatidylcholine 1,2-Dilauroyl-sn-glycero-3-phosphocholine	DLPC		R_1, R_2
Dimyristoylphosphatidylcholine 1,2-Dimyristoyl-sn-glycero-3-phosphocholine	DMPC		R_1, R_2
Dipalmitoylphosphatidylcholin 1,2-Dipalmitoyl-sn-glycerol-3-phosphocholine	DPPC		R_1, R_2
Distearoylphosphatidylcholine 1,2-Distearoyl-sn-glycero-3-phosphocholine	DSPC		R_1, R_2
Dioleoylphosphatidylcholine 1,2-Dioleoyl-sn-glycero-3-phosphocholine	DOPC		R_1, R_2
Palmitoylphosphatidylcholine 1-Palmitoyl-sn-glycero-phosphocholine	Mono-PPC		R_1 R_2
Palmitoyloleoylphosphatidylcholine 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine	POPC		R_1 R_2
Palmitoyllinoleoylphosphatidylcholine 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine	PLPC		R_1 R_2
Phosphatidylethanolamine	PE		[a]
Sphingomyelin	SM		[a]
Phosphatidylglycerol	PG		
Distearoylphosphatidylglycerol 1,2-Distearoyl-sn-glycero-3-phosphoglycerol	DSPG		R_1, R_2
Dipalmitoylphosphatidylglycerol 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol	DPPG		R_1, R_2
Phosphatidylserine	PS		

Table 1 (*Continued*)

Compound	Symbol	Structure	*R*
Dipalmitoylphosphatidylserine 1,2-Dipalmitoyl-sn-glycero-3-phosphoserine	DPPS		R_1, R_2
Phosphatidic Acid	PA		[a]
Phosphatidylinositol	PI		[a]
Cardiolipin	CL		[a]
Cholesterol	Ch		
Dicetylphosphate	DCP		

[a] R_1, R_2 depend on source.

ranges between several tens and thousands of nanometers. With diameters from ~25 to ~100 nm they are usually termed "small unilamellar vesicles" (SUVs) and from ~100 nm to ~1 μm "large unilamellar vesicles" (LUVs). "Giant unilamellar vesicles" (GUVs; 20–150 μm) are formed rather from bipolar tetraether lipids like those occurring in *Archaea* than from esters like the phospholipids [1]. *Archaea* are microorganisms which are adapted to extreme environmental conditions like high temperatures, low pH or absence of oxygen.

Phospholipid liposomes have been utilized since about two decades in liquid chromatography (LC) and became popular for the investigation of interactions between ligands and biomembranes (for recent reviews, see refs. [2,3]). In electrically driven separation methods in the capillary format liposomes were less frequently used and investigated. However, since their introduction into these methods [4,5] they have raised two-fold interest; on the one hand, they were considered attractive separation media especially as models for the partition of analytes into cell membranes. On the other hand, capillary electrophoresis (CE) turned out to be an effective tool to investigate the properties of liposomes as analytes, like their charge and size distribution, or their interaction with ligands. It is interesting that most types of liposomes carry a charge (normally they are anions) even when they are composed of neutral compounds or of zwitterionic lipids in their isoelectric range, at pH values where the opposite charges should compensate each other. In this respect the liposomes behave similarly to other colloidal particles composed of apparently electrically neutral compounds like polyethylene, polyvinylchloride or elementary gold [6].

The use of liposomes as stationary or pseudo-stationary phase in electrically driven techniques has some advantages as compared to column chromatography. The setup of the separation systems is much less complicated; in liquid chromatography, the liposomes have to be attached to the stationary phase. This is not the case in electrokinetic chromatography (EKC). Here, the vesicles are freely dispersed in solution without steric restrictions thus better mimicking conditions such as they prevail for lipid vesicles within cells. Similar to micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatography (MEEKC) consumption of sample and chemicals is very low as compared to classical column LC. For reviews on the use of liposomes in separation techniques and on their characterization and analysis by CE see refs. [2,3,7,8].

First the application of phospholipids as pseudo-stationary phase added to the background electrolyte (BGE), and as real stationary phase, when they form a coating layer at the capillary

wall is discussed. In both cases, separation of the analytes is based on their partition between the aqueous buffers and the lipid membranes; in principle the system is a chromatographic one. Migration is due to electrokinetic movement, by electrophoresis for charged analytes and dispersed charged vesicles, and by the superimposed electroosmotic flow (EOF). Then, work dealing with the characterization of liposomes by CE is discussed. CE analysis of liposomes is possible because in many cases the vesicles exhibit an electric charge as mentioned above. Finally, affinity electrophoresis in the broadest sense involving liposomes will be discussed and at the end of this contribution a separate section is devoted to peptides and proteins. It is obvious that these topics overlap in some cases.

2. Liposomes for electrically driven capillary chromatography

When liposomes are used as lipophilic chromatographic phase, two methods are differentiated in the literature according to the arrangement of this phase. In both methods the mobile (aqueous) phase is moving through the separation system by the EOF. In capillary electrochromatography (CEC) the stationary phase is really fixed in the system, either as a packed bed, a monolithic phase, or as coated layer at the inner capillary wall. In EKC the lipophilic phase is suspended in the aqueous mobile phase (or vice versa) in form of micelles, microemulsions or other particles forming a pseudo-stationary phase. These particles are normally electrically charged and move thus with an own electrophoretic velocity under the influence of the applied electric field. With respect to the separation principle being a chromatographic one it is not a pre-requisite that the one phase is fixed, it is essential that they migrate in the aqueous phase with the EOF. The analytes are electrically driven; if the compounds are uncharged they migrate with the EOF. Separation is possible due to the different partitioning of the analytes between the two phases. Note that the separation of charged analytes in these electrically driven systems can be described similarly by taking into account their own electrophoretic migration. In this section we will discuss analyte separation rather than the physico-chemical parameters it is based on; distribution coefficients and binding constants are the topic of the separate Section 4.

2.1. Liposomes as pseudo-stationary phases

Nakamura et al. [9] used liposomes as pseudo-stationary phase in EKC for the separation of hydrophobic compounds. Liposomes consisting of anionic and cationic lipids were prepared by Foley and co-workers [10]. The vesicles were made from *n*-dodecyltrimethylammonium bromide (DTAB) and sodium dodecyl sulphate (SDS). These detergents, either pure or as mixture, form micelles. However, at a mass ratio of 40/60 of DTAB/SDS and at a total surfactant concentration of 1% (w/v) the detergent mixture forms vesicles of about 100 nm diameter, with the cationic groups directed towards the interior, and the anionic groups towards the exterior of the vesicle. The authors compared these vesicles with SDS micelles and mixed micelles formed by SDS and DTAB with respect to detergent ratio, phase ratio, elution range and selectivity, hydrophobicity, etc. The vesicular systems showed a larger elution window as compared to the micelles.

LUVs formed from two different zwitterionic phospholipids, either with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or with 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) as main constituent, were prepared by Wiedmer et al. [11] and their retention characteristics were evaluated with corticosteroids as reference compounds. A second, anionic lipid (PS; cardiolipin (CL); phosphatidylglycerol (PG); or phosphatidic acid (PA)) was present at up to 30% in the liposome preparations. Running buffer was 50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) at pH 9. Boric acid buffer was found to be unsuitable due to the interaction of borate with the steroids: they were retained even in the absence of liposomes. Relative migration times, defined as t_m/t_0 (t_m is the migration time of the analyte, t_0 that of an EOF marker) were given; they increased with total lipid concentration and decreased with the POPC/CL ratio. The effect of the anionic head group (from PG, PA, PS or CL, respectively) was investigated at different temperatures. Separation of the corticosteroids increased with increasing negative charge of the vesicles.

Steroid hormones could be separated using liposomes made from POPC and POPC/cholesterol (Ch) (80/20 mol%). The electrophoretic (anionic) mobility of the vesicles with particle diameters of around 120 nm were small (between 2 and 13×10^{-9} m^2 V^{-1} s^{-1}) and changed with buffer type (at constant ionic strength of 20 mM and 25 °C) [12]. The EOF was dependent on the coating of the capillary. Retention of analytes correlated with the results obtained by monolayer penetration measurements. With similar liposomes (POPC/PS, but no Ch) phenols and steroids were separated [13].

A similar comparison as in ref. [10] was made with three surfactant vesicles and one phospholipid vesicle [14]. The authors related properties like particle diameter, mobility, retention and migration window, separation efficiency and selectivity to those of common MEKC systems with SDS. Two types of surfactant vesicles were formed from non-stoichiometric aqueous mixtures of the oppositely charged and single-tailed surfactants cetyltrimethylammonium bromide (CTAB) and sodium octyl sulfate (SOS) at a 30/70 molar ratio, and of *n*-octyltrimethylammonium bromide (OTAB) and SDS at a 70/30 molar ratio. Other surfactant vesicles consisted of double-tailed bis(2-ethylhexyl)sodium sulfosuccinate (AOT) in 10% MeOH, and phospholipid vesicles were made from POPC/PS in a ratio of 80/20. The vesicles had mean diameters of between 76 and 108 nm, and mobilities of about 35×10^{-9} m^2 V^{-1} s^{-1} (with the exception of OTAB/SDS, which exhibited a mobility of only 17×10^{-9} m^2 V^{-1} s^{-1}). Interestingly, all vesicles were negatively charged, although the OTAB/SDS vesicles were prepared with the cationic surfactant added in excess. The phase ratios of the pseudo-chromatographic systems were between 10^{-2} and 10^{-3} (with surfactant concentrations between 0.5 and 1.8%, w/v). CTAB/SOS vesicles formed spontaneously in aqueous solution. A constant particle size was obtained after 14 h of growth as determined by dynamic light scattering. For a moderately retained analyte (the retention factors were not given)

plate numbers between 60,000 and 130,000 were measured. The same migration order for positional isomers (nitrotoluenes) was observed for the vesicle systems, but it differed from that seen when SDS micelles were used. Interpretation of the retention data based on linear solvation energy relationship (LSER, see Section 4) revealed that hydrogen bond acidity and cohesiveness were the most relevant parameters. In previous work of this group, similar systems (CTAB/SOS at 80/20 mol% and AOT in 10% MeOH) were used to correlate retention factors to P_{OW} (the partition constant between octanol and water) for a number of organic standard compounds, pesticides and organic acids [15]. The results were based on LSER.

The same group investigated the influence of organic solvents added to the BGE on particle size, retention of neutral analytes, methylene and shape selectivity, and based the interpretation of the change in retention on LSER analysis [16]. So-called class I (at 0.5%, v/v) and class II solvents (at up to 15%, v/v) were added to the BGE, consisting of 1.8% (w/v) CTAB/SOS in 10 mM *N*-(2-hydroxyethyl)piparizine-2′-(2-ethanesulfonic acid) (HEPES) buffer at pH 7.2. Class I modifiers, polar organic compounds like alkylpolyols, are absorbed in the vesicle and change thus the partition properties for the analytes. From this class the authors applied 1-butanediol, 1,2,6-hexanetriol, glycidol, and 2-amino-1-butanol. Class II modifiers change physical properties of the bulk liquid phase like the dielectric constant, and affect in this way analyte partition. Solvents like methanol or acetonitrile belong to class II; acetonitrile was chosen in this paper. It was found that all modifiers decrease the methylene selectivity, α_{CH_2}. The methylene selectivity can be derived from the slope of the curves relating the retention factor, k, of a series of homologues versus their carbon number, n_C. For this purpose, alkylphenones are often taken. The according regression can be expressed by

$$\log k = \log \alpha_{CH_2} n_C + \log \beta \quad (1)$$

$\log \beta$ is the intercept of the regression line. It was further found that the shape selectivity is decreased by the class II modifier. Shape selectivity is the selectivity coefficient which relates the retention factors of two positional isomers, 1 and 2, according to k_1/k_2. The class II modifier acetonitrile, on the other hand, did not affect shape selectivity. Based on LSER analysis of the retention data it was concluded that cohesiveness and hydrogen bond acidity play the major role when acetonitrile was the modifier. With class I solvents cohesiveness is important as well, but hydrogen bond basicity is decisive. In a recent paper [17] the authors compared the effect upon changing the counter-ion of the cationic detergent from bromide to chloride. The size of the vesicles changed from an average diameter of 85 to 96 nm. Class I and class II modifiers had similar effects on elution range, methylene selectivity, and efficiency. LFER analysis suggested a difference in solute–vesicle interaction due to the counter-ion.

The elution range for EKC with particles containing chiral *N*-dodecoxycarbonylvaline (DDCV) was increased by applying mixed vesicles with an oppositely charged detergent, CTAB [18]. The vesicle-based separation system finally consisted of 1.4% (w/v) CTAB/DDCV (30/70 mol%) in 35 mM CHES buffer, pH 8.5. Compared to mixed micelles consisting of the DDCV and SDS, a larger elution range, higher methylene and shape selectivity was observed. Interestingly enantioselectivity was completely lost in the vesicle system compared to the micellar one, indicating a different separation mechanism in both EKC systems.

The aggregation behaviour of a novel detergent, sodium *N*-(4-dodecyloxybenzoyl)-L-valinate (SDLV), which differs from that used in ref. [18] by substitution of the carbonyl by a benzoyl group, was investigated by dynamic light scattering, microscopy, fluorescence probe and surface tension methods [19]. It spontaneously forms vesicles with 30–70 nm diameter in aqueous solutions. It was used as chiral selector for EKC [20]. The atropisomers of binaphthol, binaphthyl diamine and binaphthol phosphate, and the enantiomers of Tröger's base and benzoin were separated in borate buffer (at pH 9.7 and 10.3) with 2–5 mM SDLV.

Retention factors of drugs in liposome systems composed of POPC and PS (in a molar ratio of 80/20, suspended in phosphate buffer of ionic strength 0.05 M at pH 7.4) were compared with different micellar and microemulsion systems with respect to the $\log P_{OW}$ values and membrane permeability [21]. Literature data on the permeability of monolayers of the human intestinal epithelial cell line Caco-2 and of the intestinal segment of rat ileum and rat colon for 20 compounds (including 10 β-blockers) were taken as reference. Although the correlations were not high (the highest correlation coefficient was 0.88) the best correlation with membrane permeability was observed for the liposomal system.

2.2. Immobilized phospholipids as stationary phases

Phospholipids and liposomes have been applied as stationary phase in LC for a long time; see e.g. the recent review article of Wiedmer et al. [2]. For their use in CEC phospholipid layers can be immobilized on the capillary wall where they form a hydrophobic stationary phase. There is no principal disparity between electrokinetic and pressure driven chromatography concerning separation selectivity with regard to neutral analytes; the difference lays in the zone dispersion effects. However, for ionic analytes it is obvious that the selectivity of the two methods differs due to the additional contribution of the electrophoretic migration in CEC.

A method to immobilize liposomes in the capillary for CEC was proposed by Yang et al. [22] who made use of the high affinity avidin/biotin system; first, the silanol groups of the fused silica capillary were reacted with 3-aminopropyltriethoxysilane. The resulting aminopropylsilica surface was treated with glutaraldehyde to obtain an aldehyde-activated surface, to which avidin was coupled. Finally, unilamellar liposomes assembled from PC and 2 mol% biotinylated polyethylene were immobilized to this surface. The vesicles had 40 or 90 nm mean diameter. The smaller vesicles contained ∼80, the larger ones ∼800 biotinylated polyethylene molecules at the outer surface. The authors argue that repeated coating finally resulted in up to 15 liposome layers on the wall.

Örnskov et al. [23] attached derivatized agarose carrying positively charged quaternary ammonium groups electrostatically to the silica surface simply by rinsing the capillary with the solution. The negatively charged liposomes were then immobilized to the agarose via electrostatic interaction by a subsequent flush. Drugs as analytes were retained due to the stationary phase as concluded from the comparison with their migration in non-coated capillaries. The migration sequence of the analytes was in agreement with their lipophilicity expressed by log P_{OW}.

Cunliffe et al. [24] coated the wall of the silica capillary with 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) in order to prevent protein adsorption in CE. The lipid was not intentionally applied as stationary chromatographic phase. CE of both cationic and anionic proteins resulted in highly efficient separations, indicating a reduction of adsorption, especially for the cationic analytes. However, incomplete protein recovery, together with some tailing of the electrophoretic peaks of the cationic proteins pointed to residual adsorption.

Riekkola and co-workers [25] has published a series of papers describing methodologies to produce immobilized liposome layers for CEC. In the first paper, they presented a simple method for coating the capillaries. LUVs with a diameter of about 100 nm consisting of POPC with different proportions of PS and Ch were prepared as usual by extrusion; they form a bilayer on the silica surface by simple rinsing. The best separation of uncharged steroids and column stability were obtained with anionic POPC/PS (80/20 mol%) liposomes. HEPES buffer turned out to be most favourable for the separation when used in the coating procedure and in the BGE. The favourable role of HEPES was confirmed in ref. [26]. It was shown that hydrophobic interactions between analytes and the negatively charged phospholipid coating (PC/PS) are important for the migration of charged analytes. Other piperazine-based compounds, i.e., *N*-(2-hydroxyethyl)piperazine-*N*′-(2-hydroxypropanesulfonic acid) (HEPPSO), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and piperazine-*N*,*N*′-bis(hydroxypropanesulfonic acid) (POPSO), at pH 7.4 were evaluated as solution constituents for liposome coating and as buffering compounds for the separation of the analytes; the phospholipid coatings again consisted of PC and PS. The quality of the coating was evaluated via separation of five steroids used as neutral model analytes. Similar to HEPES, addition of small diamines (ethylenediamine, diaminopropane, bis-tris-propane) to the liposome solution improved the coating quality [27] (see Fig. 1). Improved separations were related to an increase in packing density of the anionic phospholipids caused by the linear diamines. It was observed that in contrast to the diamine, buffers like phosphate may have negative effect on coating formation. Finally, it was demonstrated that Ca-ions can most favourably be used as substitute for HEPES for the stabilization of the phospholipid coating [28,29]. Application of Ca^{2+} in the coating procedure as well as its presence in the separation buffer containing 3 mM POPC/PS vesicles (at a ratio of 80/20 mol%) led to an improvement in separation of steroids and phenols. In a recent paper Hautala et al. [30] investigated the stability of the phospholipid layer as a function of the buffer pH used during the coating procedure, and for electrochromatographic separation. The liposome solution consisted of 3 mM PC/PS in molar ratio of 80/20; buffer constituent was HEPES. The authors argue that the extent of the attachment of the coating to the fused silica surface is connected to the protonation of the amines of both, the phospholipids and HEPES. The authors found that, as in ref. [28], Ca^{2+} plays an important role in stabilization of the layer. Separation of five steroids was investigated, and relative migration times of the analytes (related to the residence time of an EOF marker) were given. Because the steroids employed were neutral compounds, the migration times can easily be converted into retention factors, k_i. It can be deduced that the k_i values are significantly larger in systems containing Ca^{2+}. The coating was stable at a pH between 4.5 and 8.0. At pH 10.8 the phospholipids leaked out.

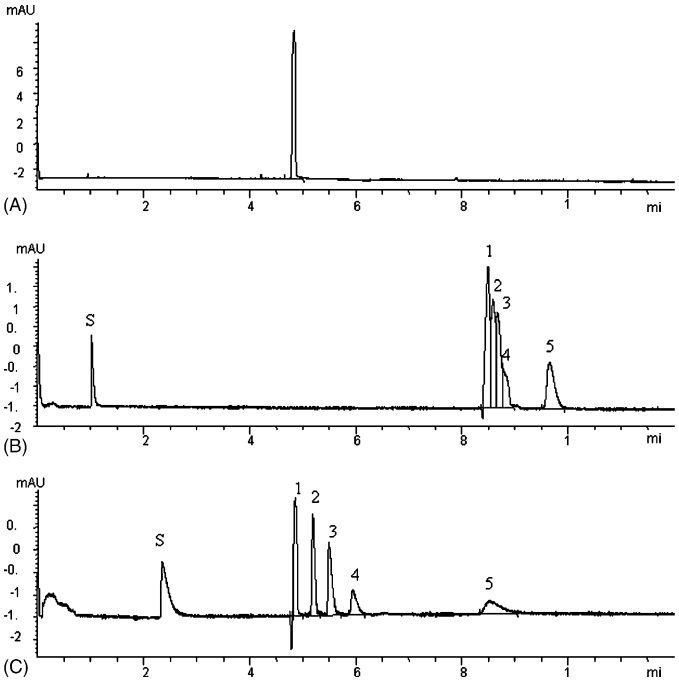


Fig. 1. Effect of the liposome coating on the separation of steroids. BGE in all cases 20 mM Tris, pH 7.4. (A) No coating; (B) PC/PS coating using 10 min preflush with 5 mM 1,3-diaminopropane (DAP) before coating with PC/PS (80/20 mol% 1 mM solution); (C) capillary coating as in (B); but 5 mM of DAP added to the liposome solution. Sample aldosterone (1), androstenedione (2), testosterone (3), 17α-hydroxyprogesterone (4), progesterone (5); voltage 20 kV, capillary length 60/51.5 cm. (S) Indicates a system peak. From ref. [27] with permission.

From the measured migration time in untreated and in liposome coated capillaries retention factors and free energies of interaction were derived for a number of drugs [31]. The analytes (salicylic acid, acetylsalicylic acid, ketoprofen, warfarin, phenytoin, propranolol) were ionized at the pH 7.5 of the BGE. The capillary was coated with a layer of POPC. The reported capacity (retention) factors were between 0.7 and 5.7. As the phase ratio was unknown, distribution coefficients were not determined. A significant loss in separation selectivity for the liposome-modified capillary, as compared to the uncoated one, was observed and related to the interaction between analyte and stationary phase. A similar investigation was carried out with POPC liposomes not bound to the wall, but introduced into the capillary as a suspension plug [32].

3. Analysis and characterization of liposomes by capillary electrophoresis

A number of papers deal with CE in the context of liposomes but not with analyzing the vesicles explicitly. They describe CE as a method for determining drugs encapsulated in liposomal formulations, mainly by measurements of the concentration of free drug by direct analysis [33–37], and the total concentration after disruption of the liposomes, e.g. by solubilisation with detergents. Stability of the liposomes and drug leakage was determined by direct injection of the formulations as long as drug and vesicles could be resolved.

In one of the first papers dealing with CE of liposomes Roberts et al. [5] investigated the size distribution of vesicles consisting of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), dicetylphosphate (DCP), and Ch. A cationic membrane dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, DiI-$C_{18}(C_5)$, served for the detection of the vesicles at 650 nm. The authors related the width of the electrophoretically measured liposome peak to the size distribution measured by laser light scattering. The average particle size was 355 nm. The relatively wide size range (expressed by the standard deviation) as derived from laser light scattering was ±210 nm. The mean electrophoretic mobility of the anionic vesicles was $39.3 \times 10^{-9}\,m^2\,V^{-1}\,s^{-1}$ but no values for the distribution of the mobilities of the liposomes are given. The authors related peak dispersion not to longitudinal diffusion; they assumed that the particles are too large as to exhibit a significant diffusional mass transport during the residence time in the capillary. The diffusion coefficient, D, was $1.5 \times 10^{-12}\,m^2\,s^{-1}$, which is about two to three orders of magnitude smaller than that of, for example, compounds with one benzene ring [38]. This means that, according to the Einstein equation for one-dimensional diffusion ($\sigma_z = \sqrt{2Dt}$) the spatial peak width σ_z, after the same migration time, t, is by more than one order of magnitude smaller for the liposome than for a small organic molecule; in fact it should be negligible. For the liposome a charge of −821 was calculated, which seemed low taking into account the large number of embedded ionic compounds. However, the number was considered as reasonable due to the non-stoichiometric ratio of cationic and anionic lipid components of the vesicle. The liposomes were lysed upon reaction with the surfactant *n*-octyl-β-D-glucopyranoside, either off-line or in the capillary by injection of plugs of solutions containing liposomes and detergent. Like in the initial liposome preparation spikes were observed in the electropherograms in increasing number and these were accompanied by the disappearance of the liposome peak upon increasing the concentration of the detergent. The spikes were related to aggregates of the liposomes, and to precipitated lipidic particles formed after disruption of the bilayer membrane.

Kawakami et al. [39] analyzed, by CE, the homogeneity of liposomes composed of DPPC and 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) in a 10/1 molar ratio. The authors related the particle size to migration time. With non-charged UV-absorbing molecules embedded in the membrane, the heterogeneity of the membrane composition of the vesicles of a certain preparation even when monodispersed was derived from the ratio of the UV signals at two different wavelengths. The migration time of the vesicles of about 100 nm diameter made from DPPC/DSPG was measured. The vesicles were most probably unilamellar as they were produced upon extrusion. The migration times showed a discontinuity when the temperature of the CE system was increased [40]. This discontinuity was related to the transition temperature from the gel-like to the liquid crystal phase. It was assumed that softening of the mem-

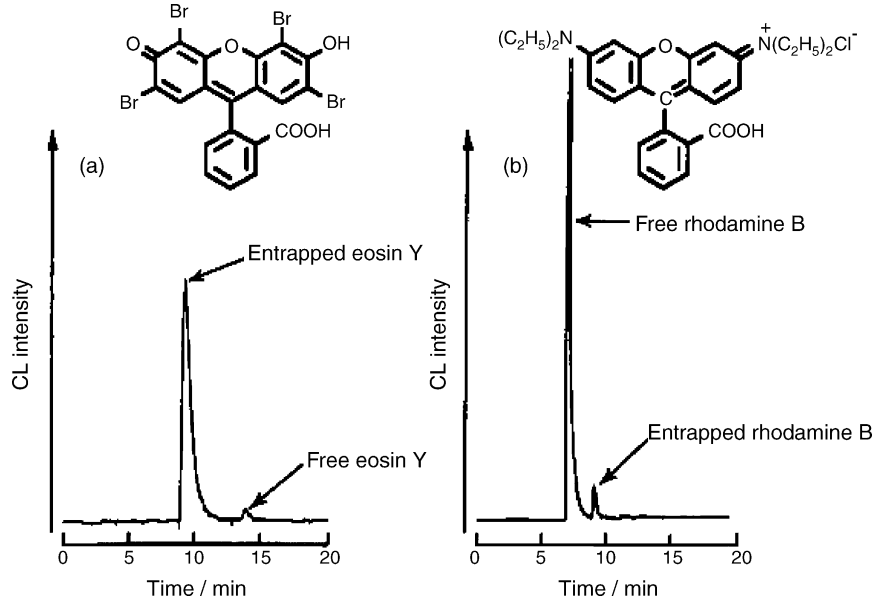


Fig. 2. Electropherograms of liposomes with entrapped dye and free dye. (a) Eosin Y; (b) rhodamine B. SUVs were prepared from DPPC. Conditions: fused silica capillary, 70 cm length, 50 μm I.D.; voltage 15 kV; BGE for separation 10 mM sodium carbonate buffer (pH 9.0); chemiluminescence detection, reagent mixture, 50 mL of 1 mM bis(2,4,6-trichlorophenyl)oxalate (TCPO) acetonitrile solution and 288 μL of 30% (w/w) aqueous H_2O_2. From ref. [41] with permission.

brane, e.g. by addition of Ch or short-chain lipids, would lead to more elongated liposomes in the electric field during migration, accompanied by a reduced frictional force and a higher mobility. Substitution of DPPC by Ch shifted the discontinuity point of the curve (plotting migration time versus viscosity) to higher viscosity and lower temperature, respectively, which was as expected from the decreased membrane rigidity.

Liposomes made from DPPC and 1,2-dipalmitoyl-sn-glycerol-3-phosphoserine (DPPS) containing dyes (eosin Y or rhodamine B) were analyzed by Tsukagoshi et al. by CE and detected by chemiluminescence upon reaction of the dyes with bis(2,4,6-trichlorophenyl)oxalate (TCPO) and H_2O_2 [41] or peroxyoxalate [42,43] at the capillary outlet. The dyes were trapped in the vesicles during the preparation of the liposomes. The stability of the vesicles and the permeation of the dye could be monitored by the appearance of the peak of the free dye emerging in the electropherograms (see Fig. 2).

With an ingenious approach Arriaga and co-workers [44] were able to measure the size and mobility distribution of individual liposomes. The authors separated multilamellar vesicles composed of PC, PS, PE and Ch in coated capillaries and detected the individual particles by laser-induced fluorescence (LIF) using an Ar-laser (488 nm). The output of the detector photomultiplier was passed through a low pass analog filter with RC of 10 ms, which allowed the detection of the single events (Fig. 3). The particles exhibited an average diameter of 1.1 μm, the standard deviation of the size distribution was 0.2 μm and their size range was between 0.8 and 3 μm. The mobilities of the individual anionic particles were approximately Gaussian distributed and between about 20 and 40×10^{-9} m^2 V^{-1} s^{-1}, with an average of 30×10^{-9} m^2 V^{-1} s^{-1} and a standard deviation of 3×10^{-9} m^2 V^{-1} s^{-1}. The authors calculated the vesicle volumes to about 1.4 fL; the volume distribution was clearly not Gaussian, it was rather a steep Poisson-like distribution with the maximum number of particles with about 0.2 fL volume (see Fig. 4 in ref. [44]). According to the authors the distribution of the mobility most probably originated from different surface charge densities of the membrane and/or polydispersity of the liposomes (see also ref. [45]).

For liposomes consisting of PC/PG/Ch in various ratios Radko et al. [46] found that the electrophoretic migration was directly related to particle size (ranging from 125 to 488 nm in mean diameter). Investigations were carried out at different ionic strength. Size-dependent migration was a function of κR (κ^{-1} is the thickness of the electric double layer which depends on the ionic strength of the buffer; R is the liposome radius). The mobility of the liposomes thus depends on κR and on the surface charge density, in accordance with the Overbeek–Booth electrokinetic theory. The authors related the size-dependent electrophoretic separation of the liposomes mainly to the relaxation effect caused by the finite relaxation time needed to re-establish the ion cloud upon the movement of an ion.

Wiedmer et al. [13] measured electrophoretic mobilities of liposomes by CE and determined their size by dynamic light scattering. The liposomes were composed from binary mixtures of POPC with CL, PG, PA or PS, and unilamellar vesicles were produced from multilamellar vesicles (lipid concentration about

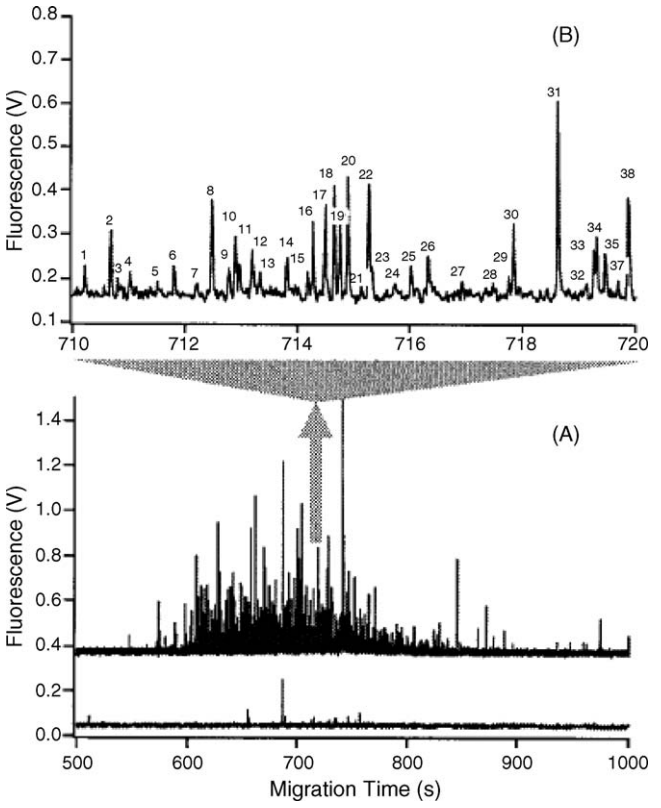


Fig. 3. Electropherograms of a liposome suspension. (A) Top record: electropherogram of the original liposome suspension five-fold diluted (offset +0.15 V). Bottom record: 100-fold dilution of liposomes not containing fluorescein. (B) Expanded migration window from 710 to 720 s in the electropherogram of the five-fold dilution (A, top record). Liposome composition PC, PS, and PEA and Ch in a molar ratio of 47.3/2.3/42.9/7.5. Separation: -200 Vcm^{-1} in 250 mM sucrose, 10 mM HEPES, pH 7.5 in a 50 μm I.D. poly(acryloylaminopropanol)-coated capillary. Fluorescence detection: Ar-laser, 20 mW, 488 nm excitation, 535 ± 17nm band-pass, 1000 V PMT bias. Data acquisition: 50 Hz. From ref. [44] with permission.

3–4 mM) in the usual way by extrusion. The mobilities of the anionic particles were about the same for the same ratio between the second lipid and POPC; in a 50 mM CHES buffer with pH 9 mobilities were about 40×10^{-9} m^2 V^{-1} s^{-1}. They increased with increasing concentration of PS. Particle diameters were independent of composition, namely about 110–120 nm. A given liposome preparation migrated significantly slower in borate buffer as compared to other buffers with the same pH of 9.0.

The electrophoretic mobilities of liposomes (consisting of PC, PA and Ch) with a pH gradient between the lumen and the outer solution were studied by Phayre et al. [47]. The particle diameter was between 130 and 170 nm (as measured by dynamic laser light scattering). The pH difference between the luminal solution (pH_i) and the outside buffer (pH_o) was 1.4 units with the higher pH either inside or outside. These liposomes were compared with vesicles having the same pH of 7.4 on both sides of the membrane (Fig. 4). A significant difference in mobility was found between all three types of vesicles. Their mobilities were in a range between 29 and 37×10^{-9} m^2 V^{-1} s^{-1} and decreased in the sequence (pH_i/pH_o): 8.8/8.8 > 7.4/7.4 > 7.4/8.8 > 8.8/7.4. The mobilities could not be related to the different degree of ion-

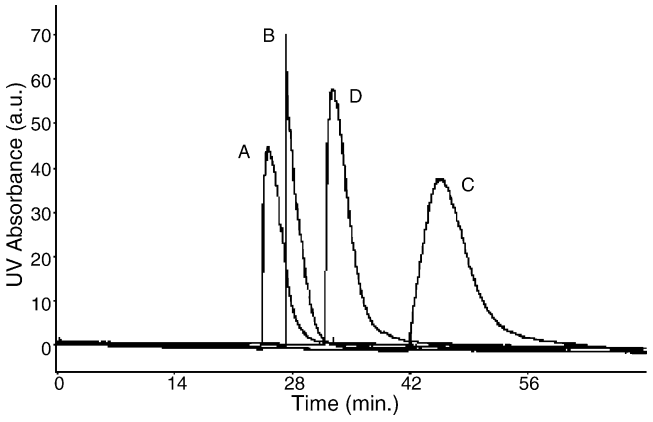


Fig. 4. Electropherograms of liposomes with different pH interiors and exteriors: (A) 8.8 in, 8.8 out; (B) 7.4 in, 7.4 out; (C) 8.8 in, 7.4 out; (D) 7.4 in, 8.8 out. Liposome made from PC and PA (10:1 molar ratio), and 20% (mol/mol) Ch. Liposome diameters between 130 and 170 nm. Capillary 62/77 cm length 50 μm I.D., coated with BRIJ35. BGE: 2 mM tricine, 15 mM potassium sulfate titrated to pH 7.4 or 8.8 with 1 M sodium hydroxide, 0.001% (w/v) BRIJ 35 added. Voltage −25 kV; UV detection at 214 nm. From ref. [47] with permission.

ization due to acid–base equilibria. It was concluded that more sophisticated models were needed, taking the membrane as electrical capacitor on the one hand, and considering the relaxation effect of ion migration, on the other hand.

In continuation of previous work, Hayes and co-worker [48] investigated the discrepancy between the electrophoretic behaviour of liposomes under various experimental conditions and that predicted by electrokinetic theories. In extension of classical theories for rigid, spherical colloidal particles the variation of ion densities and electric potentials within the ion atmosphere normal to the surface of the large particle were considered together with the deformation of the liposomes to spheroid particles with prolate shape. However, the predicted mobilities were always smaller than those found experimentally, although the shape of the curves depicting the reduced mobility as function of κR agreed with the theory. The authors related these deviation to the unique properties of the liposomes, namely to their deformability and their susceptibility to field-induced polarization. In their most recent work [49] these authors improved the theoretical prediction of electromigration of colloids, taking into account multipole effects, deformability, polarisability and mobile surface charges.

4. Distribution coefficients and binding constants

Distribution coefficients between the lipophilic phospholipid vesicles and the aqueous phase can be determined by CE in the same way as with MEKC or MEEKC. As described in Section 2.1, in a number of papers Foley and co-workers [10,14–18] related $\log k$ to $\log P_{OW}$ values and applied LSER to interpret the interactions of the solutes with the liposomes. Khaledi and co-workers [50] made an investigation of the factors that are responsible for partitioning of analytes between the aqueous phase and vesicles formed from dihexadecylphosphate (DHP), an anionic double-chain surfactant. The authors applied LSER based on the descriptor values of 41 uncharged test solutes. The LSER model quantifies the contribution of individual interactions on the retention factor, k, according to

$$\log k = vV + bB + aA + sS + eE + C \quad (2)$$

V is the McGowan volume, B the hydrogen-bond acceptor basicity, A the hydrogen-bond donor acidity, S the dipolarity/polarisability, and E is the excess molar refraction. The coefficients v, b, a, s and e are relative measures for the interaction of the analytes with the pseudo-phase compared to the aqueous phase. v is a measure for the difference in cohesive energy between the aqueous and the pseudo-stationary phase, b for the H-bond donor strength, a for the H-bond acceptor strength, s for the dipolarity/polarisability and e for the interaction of the pseudo-stationary phase with n-or π-electrons of the analytes. C is the regression constant which is given by the phase ratio.

The results were compared with analyses using SDS and sodium dodecyl phosphate (SDP) with respect to $\log P_{OW}$. The authors pointed to the shortcomings of taking $\log P_{OW}$ values to describe bio-partitioning of drugs, because n-octanol is a bulk phase, whereas a membrane displays a structured environment. Therefore, it might be assumed that partitioning into a membrane would better correlate with partitioning into micelles than into a bulk liquid. However, interestingly the opposite was observed: $\log k$ values from the higher structured DHP vesicles correlate better with $\log P_{OW}$ values than with $\log k$ from micelles. A detailed analysis of the LSER results shows that size and hydrogen bond acceptor strength play the major role in partitioning between water and the SUVs. Analysis of the Gibbs free energy of transfer for the individual functional groups of the solutes from the aqueous phase into the vesicular phase shows that the energy associated with cavity formation is the main contributor. The surprising result of the better correlation of the $\log k$ values of lipid bilayer membranes with $\log P_{OW}$ than with $\log k$ values of SDS micelles was also found in liposome EKC with 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG)/DPPC and DPPG/DPPC/Ch vesicles [51]. In continuation of this work LSER was applied to interpret the retention data of neutral solutes in a system containing vesicles formed from a cationic double-chain surfactant, dihexadecyldimethylammonium bromide [52]. The vesicles were dispersed in deionised water (no buffer was used for EKC). Particles with 50 nm average diameter were formed, with the bromide ions predominantly attached electrostatically at the vesicle surface. This was concluded from their average charge, which was only 118, compared to 17,500 charges calculated from the number of surfactants per vesicle. The free energy of transfer of a functional group, R, from water to the pseudo-stationary phase, expressed as

$$\Delta\Delta G = -RT \ln \left(\frac{k_{\Phi-R}}{k_{\Phi}} \right) \quad (3)$$

was derived as well. Here $k_{\Phi-R}$ is the retention factor of the substituted benzene and k_{Φ} that of benzene. It could be demonstrated that the charge of the head group is most relevant for the interactive characteristics of vesicles.

Burns and Khaledi [53] measured the capacity (retention) factors for a number of neutral and charged analytes in sys-

tems consisting of liposomes made from DPPC, DPPG and Ch with about 40 nm diameter at pH 7.5; the partition coefficient was derived from the retention factor and the phase ratio. The latter was calculated from the partial specific volume of the surfactant, the phospholipid concentration and the critical aggregation concentration. The logarithm of the partition coefficients of the 18 investigated monosubstituted benzenes (all neutral) ranged between 1.05 and 2.99. The data obtained by liposome EKC were compared with those predicted by two methods based on quantitative structure-partition relationship (QSPR). The first method relates the partition coefficients from the liposome system to those from water/*n*-octanol. The second method uses descriptors for LSER. The good agreement between calculated and measured data illustrates that the migration properties of analytes in EKC can indeed be predicted. In addition, EKC is a fast and sensitive method with low consumption of chemicals and solvents allowing for the determination of the partition coefficients in multi-analyte mixtures. For 26 basic drugs the retention factors were determined in systems containing phospholipid vesicles made from PS, PC, PG and Ch at different ratios [54]. Buffers were HEPES, CHES, 2-(*N*-morpholino) ethanesulfonic acid (MES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and phosphate with different ionic strength, pH was 7.0 or 7.4. It was found that the retention of the cationic analytes with the negatively charged liposomes is governed by electrostatic interactions, and the ionic strength plays a dominant role at given pH. Retention data showed low correlation with log P_{OW} values. It should be mentioned that the dipolarity and polarisability of the solvation environment associated with such SUVs made from PG, PC and Ch was investigated with a series of di-*n*-alkyl-*p*-nitroanilines as solvatochromic π^* indicators in combination with size exclusion methods and photon correlation spectroscopy [55].

Khaledi and co-worker [56] also investigated the effect of the pH on the distribution of basic drugs (tetracaine, nefopam, lisocain) between the aqueous phase and negatively charged liposomes in a quantitative way by EKC. The liposomes consisted of PC, PG and Ch. Partitioning of the neutral and the cationic form, both analyte species being in acid–base equilibria, between the two phases was considered. From the retention data and based on fundamental thermodynamics the distribution coefficients of the species, and their particular fractions as a function of the pH were derived. The curves have the typical sigmoid shapes. Partition coefficients were between 46 and 1406 M^{-1} for the cations, and between 20 and 360 M^{-1} for the neutral solutes. Moreover, the authors were able to derive, from the retention data, the shifts in pK_a of the solutes caused from their interaction with the lipid bilayer. When compared to water, the pK_a values changed by between 0.05 and 0.47 units.

Interactions between cationic liposomes commercially employed for drug delivery and a fluorescein conjugated 2′-*O*-methyl-phosphorothioate (Me-PTh) antisense oligonucleotide were studied and binding constants were derived from the change of the mobility of the oligonucleotide upon modifying the liposome concentration in the BGE [57]. The antisense oligonucleotide was taken up from HeLa cells in a liposome concentration dependent manner and interfered specifically with mRNA of an aberrant luciferase reporter gene. As a result of this interaction, luciferase activity was restored. That way, the concentration of delivered antisense oligonucleotide as well as its corresponding gene expression was determined for two liposome formulations (Lipofectamine and Escort).

Liposomes were chosen as models for the affinity of drugs towards low density lipoprotein (LDL) [58]. Total binding affinities ("*nK* values") of verapramil and propanolol for liposomes were determined by frontal analysis. *nK* was calculated from C_t, the total drug concentration, from C_u, the unbound drug concentration, and from L_t, the total liposome concentration according to $nK = (C_t - C_u)/C_u L_t$. Frontal analysis by CE was carried out by co-incubation of liposomes and drug in a physiological buffer, injection of a plug of this solution, electrophoresis in the same buffer, and detection of the drug zone by UV. The height of the trapezoidal peak was taken as a measure for the unbound drug. A number of liposomes with different composition were investigated; they consisted of POPC, PLPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine), DLPC and 1-palmitoyl-sn-glycero-3-phosphocholine (mono-PPC). Significant differences in the *nK* values were found. They varied within more than one order of magnitude with values between 8.5×10^7 M^{-1} and 103×10^7 M^{-1} for verapramil and 12×10^7 M^{-1} and 178×10^7 M^{-1} for propanolol. The increase in the negative charge of the phospholipids was found more relevant for the ligand-binding affinity than the acyl-chain structure. The binding affinities as function of the liposome composition (size, surface charge) point to the great significance of the electrostatic interactions in binding of the basic drugs. It seems that liposomes bind the drugs unspecifically as in the case of LDL. The results are taken to demonstrate the suitability of liposomes as models to explain the difference of drug binding between LDL and oxidized LDL.

Marques and Schneider [59] proposed a liposome system to bind DNA in a sequence specific manner. For this purpose the authors embedded di-alkyl peptide nucleic acid amphiphiles (PNAAs) in the membrane of liposomes that consisted of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and Ch. Peptide nucleic acids (PNAs) are synthetic nucleic acid analogues which form duplexes and triplexes with complementary single and double stranded DNA, respectively. The final amount of PNAA within the membrane was determined via UV absorption. Such PNA liposomes were incubated with complementary single strand DNA oligomers. Hybridization of the components was assessed by CE. Free DNA showed a sharp peak, whereas PNA liposomes rendered a broad signal with similar mobility. Samples incubated with complementary DNA showed both signals. The broad peak, however, shifted its mobility, which strongly indicated duplex formation. From the reduced area of the DNA peak it was derived that almost all PNA formed a duplex with complementary 10-mers of DNA. A single mismatch within this sequence yielded no binding at all and longer oligomers showed greatly diminished binding to PNA liposomes. The sequence-specific binding of these liposomes and its possible application as biosensing tag in analytical devices was demonstrated.

5. Work related to proteins and peptides

The first paper describing the application of liposomes in a manner as micelles or oil droplets are used in MEKC or MEEKC, respectively, was published about 10 years ago [4]. Conceptually, the liposomes were to serve as models for biological membranes like in LC where they are immobilized on gels. In their paper the authors added liposomes consisting of PC (and cholate) to the BGE (25 mM phosphate, pH 7.4) and separated several charged analytes in a coated capillary after application of an electric field. They assumed that the mobility of both the EOF and the liposomes was negligible. Increased retention of the analytes was related to their interaction with the vesicles. In accordance with results obtained by immobilized-liposome chromatography, two octapeptides, the one with two cysteines replaced by serines with respect to the other, were separated with better resolution in the presence of the liposomes.

Apolipoproteins constitute the protein moiety of lipoprotein particles. Liposomes were used as models for lipoproteins such as very low-density lipoprotein (VLDL) or LDL in an affinity electrophoretic approach to study the interaction of apolipoproteins with lipids [60]. The unilamellar liposomes consisted of DMPC and were about 100–120 nm in diameter. The number of copies of apolipoprotein apo CIII (Mr 8.8 kDa) and its derived peptides (2.1–4.5 kDa) bound to a single liposome and the binding strength was derived by an equation which relates the change of the mobility of these ligands to the concentration of the liposomes in the BGE. This change is illustrated in Fig. 5 showing the electropherogram of apo CIII at five different liposome concentrations. Two assumptions were made: non-cooperative binding between apo CIII and the liposome, and the equality of the mobilities of the complex and the free liposome. The binding constant for apo CIII was $22 \times 10^3\ M^{-1}$ and those of the peptides were by factors of 2 to 3 lower, in agreement with data obtained by other methods. The number of analytes bound per vesicle was 1350 for apo CIII, and between 470 and 5200 for the peptides.

Tsukagoshi et al. [61] presented an immunoassay using eosin Y containing liposomes as a labelling reagent for human serum albumin (HSA). After introduction of a thiol group onto HSA via *N*-succinimidyl 3-(2-pyridyldithio) propionate as described by Carlsson et al. [62] the resulting HSA derivate was enabled to bind labeled liposomes covalently. That protein–liposome conjugate was then added to a definite amount of ordinary analyte HSA and subsequently incubated with antibody-immobilized glass beads to perform a competitive immunoassay. Since an excess of protein was used, HSA separated in a glass beads-bound and a free section. The reactant solution, consisting of free HSA (labeled and unlabeled), was applied to a CE-chemiluminescence detection system to remove compounds that might perturb the chemiluminescence and to detect the liposomes, respectively. At the tip of the capillary the liposomes were destroyed by organic solvents which contained the chemiluminescence reagent. In this way a chemiluminescence signal was induced by the vesicles. Based on the competitive binding reaction of the two HSA species to the glass beads, the amount of labeled HSA indicated a relationship to that of analyte HSA.

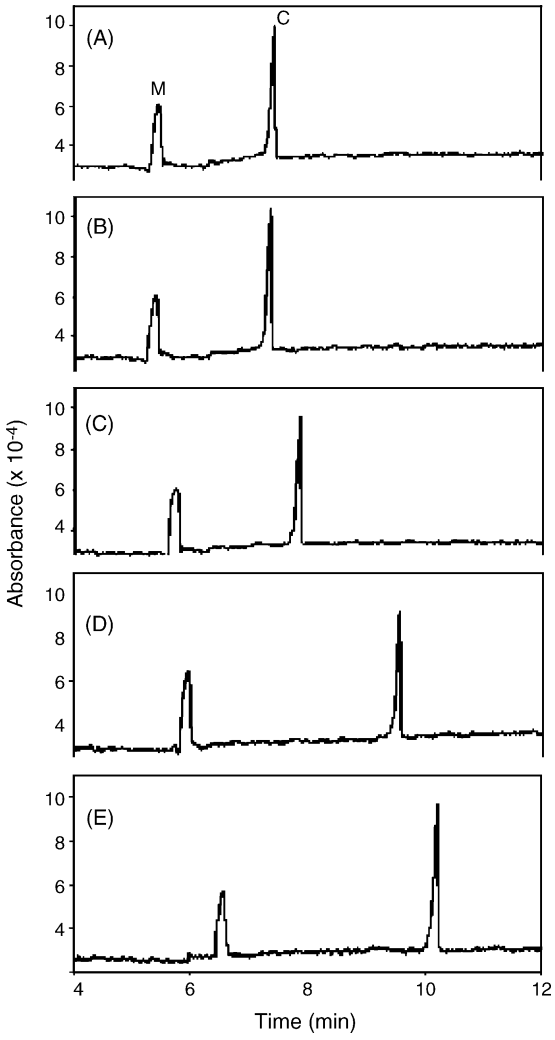


Fig. 5. Electropherograms of apolipoprotein apo CIII at different vesicle concentrations. C, apo CIII; M, internal marker. Vesicle concentrations: (A) 0; (B) 2.1×10^{-6}; (C) 1.1×10^{-5}; (D) 2.1×10^{-5}; and (E) 2.1×10^{-4} μM DMPC vesicle in phosphate-saline buffer at pH 7.4. From ref. [60] with permission.

Basic proteins (lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A) were separated in accordance with their electrophoretic mobilities by zone electrophoresis in capillaries coated with a phospholipid layer consisting of POPC in acidic [63] or neutral [64] BGEs. The same phospholipid was also used as a carrier. Coating was a condition to obtain protein peaks, as it suppressed adsorption onto the wall. Since the EOF was reduced upon coating, no significant change in the electrophoretic behaviour of the proteins was observed indicating only minor interactions between the proteins and the vesicles in solution. With the same phospholipid layer, resolution of three peptides (angiotensin I, II and III) was found to increase with increasing concentration of the phospholipids [64], indicating a chromatographic interaction of the separands with the stationary phase.

Tachibana et al. [65] proposed to insert membrane proteins into the liposomes and to determine the binding behaviour of ligands by electrochromatography. In this way they derived the binding constants between a cell wall precursor model pep-

tide $Me(CH_2)_8CO$-Gly-L-Ala-d-D-Glu-Lys(Ac)-D-Ala-D-Ala and vancomycin.

Riekkola and co-workers [66] used lysozyme, bound to the phospholipid coating, as chiral agent. The interaction of lysozyme with the lipid layer led to strong immobilization of the protein in the capillary. The layer was stabilized when the capillary wall was first coated with 1-(4-iodobutyl)-1,4-dimethylpiperazin-1-ium iodide (M1C4). The preparation of the chiral stationary phase was carried out simply by serially rinsing with the solutions containing M1C4, the liposomes, and the protein. The attached liposomes were responsible for the stereoselectivity; this was supported by the finding that the protein was not needed in the BGE for chiral separation of D- and L-tryptophan.

Bo and Pawliszyn [67] studied the dynamic process of conjugate formation between four standard proteins and vesicles consisting of PC and PC/PS (80/20 mol%) by capillary isoelectric focusing (CIEF) with whole-column imaging detection (WCID). Stable conjugates between the PC vesicles and trypsin inhibitor, β-lactoglobulin B (two conjugates), and phosphorylase b could be separated from the native proteins due to the p*I*-shift of the conjugates. Trypsinogen, on the other hand, showed an unchanged CIEF profile, which was explained by a very weak interaction with PC vesicles. Using PC/PS vesicles, the conjugates of all four proteins had lower p*I* than their native forms resulting from the acidic PS. PC/PS vesicles exhibited multiple conjugates with trypsin inhibitor (six peaks), β-lactoglobulin B (four peaks), and phosphorylase b (three peaks). This finding was explained by multiple sites interaction of the proteins with the PC/PS vesicles. The conjugates were measured at different incubation times (up to 90 min) to determine the final interaction equilibrium. The CIEF profile of trypsin inhibitor is shown in Fig. 6. In a similar paper [68] the authors investigated the phospholipids–protein interactions of seven standard proteins with PC vesicles at different PC concentration, incubation time, and incubation temperature by measuring the protein profiles with CIEF-WCID.

Receptor proteins were attached via their his_6-tag to liposomes [69]. For this purpose a lipid containing nitrilotriacetic acid (NTA) as head group was incorporated in the bilayer of liposomes consisting of POPC, PE and Ch and a short polyethylene glycol (PEG) chain for stabilisation. A Ni^{2+}–NTA complex was generated at the liposome surface, to which, in turn, the tagged proteins were bound. The two steps of complex formation could be monitored by the shift of the electrophoretic peak of the vesicle (the lumen was filled with a fluorescence dye for detection). The complex between liposome and protein was stable but was destroyed upon addition of EDTA, which removes the Ni ion and thus the receptor protein from the liposome, as evidenced by a shift of the peak to the original position.

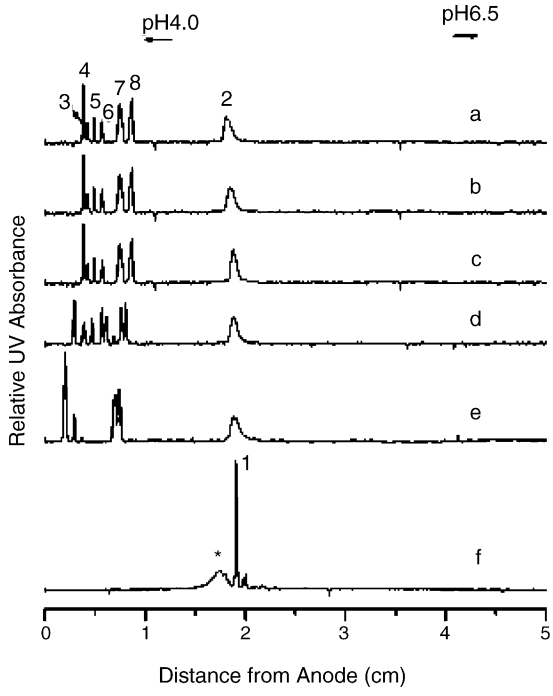


Fig. 6. CIEF profile of conjugates between liposome (PC/PS, 80/20 mol%) with trypsin inhibitor in dependence of incubation time: (a) 90 min; (b) 60 min; (c) 30 min; (d) 15 min; (e) 5 min; (f) native protein. Sample solution: 0.25% methyl cellulose, 4% pharmalytes, 10.7 mM trypsin inhibitor; 250 mM PC/PS (80/20 mol%). Catholyte, 100 mM NaOH, anolyte, 100 mM H_3PO_4. (*) Denotes degradation product of the protein. Peak 1, native protein. Peaks 3–8 PC/PS–trypsin inhibitor conjugates. UV detection at 280 nm; applied voltage 500 V for 2 min, then maintained at 3000 V; focusing time 8 min. From ref. [67] with permission.

6. Conclusion

Electrically driven chromatography with liposomes is a valuable tool to measure interactions between lipid membranes and a large variety of ligands. It needs only minute amounts of sample. The liposomes can be applied as pseudo-stationary phases in the electrically driven separation system in a simple manner, namely as stable charged colloidal or sub-colloidal constituents of the BGE.

Liposomes, attached to the wall of the separation capillary as a bilayer, form a true stationary phase in a CEC system. Some aspects should be taken into consideration concerning this methodology, independent of whether it is pressure or electrokinetically driven. The one is the slow kinetics of mass transfer in the mobile phase within the open tube due to the low diffusion coefficients in liquids (which are five orders of magnitude lower than in gases). This requires low migration velocities in order to avoid excessive peak broadening contributed from the mass transfer term. The second is the small phase ratio resulting from the thin layer of the stationary phase and the relatively large volume of the bulk mobile phase. The latter problem, which seems typical for this methodology, can be illustrated by the data published in ref. [22]. For a phase with as much as five liposome layers the amount of assembled lipid in a capillary of 40 cm length and 75 μm inner diameter is 12 nmol. The phase ratio is therefore, as small as about 10^{-3}. High partition coefficients are thus needed to obtain favourable retention factors, which should exceed values of say 1 or 2. If the retention factors are too low, and if the selectivity coefficients are not very high, a useful resolution will not be achieved. If, e.g. the selectivity coefficient is 1.1, and a low retention factor of 0.1 is assumed,

baseline resolution of 1.5 for two peaks of the same height could be achieved only if the plate number is 600,000, which is not trivial. Most systems described in the literature deliver only around 100,000 plates or less in practice. This example illustrates the limits of the CEC method. Note that here neutral analytes are considered, which are driven only by the EOF. Nevertheless, it has to be pointed out that open tubular electrochromatography has high potential with respect to separation efficiency due to the plug-like velocity profile of the ionic analytes as well as of the EOF. Taylor dispersion, as occurs in open tubes due to the parabolic velocity profile of the hydrodynamic flow, is absent.

Migration data (retention factors or electrophoretic mobilities) can elegantly be used to derive thermodynamic data like binding constants, partition coefficients or free energies of transfer. This is an interesting methodology when interactions of biomembranes with ligands like drugs, peptides, proteins or other biopolymers should be mimicked.

For the analytic characterization of the vesicles their different electrophoretic velocities when migrating in the electric field can be utilised. It should be noted that there is no direct relation between mobility and size of the vesicles. No simple theory can describe the relation between these two parameters. However, physical properties like mobilities, diffusion coefficients or charge numbers, and other characteristics such as membrane stability can be determined in an easy manner.

Acknowledgement

This work was supported by the Austrian Science Foundation (Project P15667).

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