CHARLES UNIVERSITY
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# CAPILLARY ELECTROPHORESIS FOR LIPOSOME ANALYSIS AND DRUG INTERACTION STUDIES

Dissertation thesis

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The experimental work that constitutes this Ph.D. thesis was realized between October 2020 and July 2024. Results from capillary electrophoresis were obtained in the laboratory based at the Department of Analytical Chemistry, Faculty of Science, Charles University, Prague, Czech Republic.


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I declare that all the results used and published in this thesis have been obtained by my experimental work supervised by doc. RNDr. Tomáš Křížek, Ph.D., all references are properly cited and this thesis had not been applied to obtain the same or other academic degree.

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Prague, August 16th 2024 ……..

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This dissertation thesis is based on two articles published in peer-reviewed international journals. In addition, one manuscript recently submitted.

1. **Tomnikova, A.**, Orgoníková, A., Křížek, T. (2022) Liposomes: preparation and characterization with a special focus on the application of capillary electrophoresis. *Monatshefte fur Chemie - Chemical Monthly*, *153*, 687-695.
2. **Šimonová, A.**, Píplová, R., Balouch, M., Štěpánek, F., Křížek, T. (2024) A comprehensive study on capillary surface modifications for electrophoretic separations of liposomes. *Monatshefte fur Chemie - Chemical Monthly*.
3. **Šimonová, A.**, Balouch, M., Štěpánek, F., Křížek, T. (2024) Investigating drug-liposome interactions using liposomal electrokinetic chromatography. Manuscript submitted to *Analytical and Bioanalytical Chemistry*

# ABSTRACT (EN)

The first part of this dissertation thesis investigates the possibilities of analysis and characterization of liposomes using capillary electrophoresis. Our primary objectives were to identify suitable background electrolytes and optimize experimental conditions to minimize liposome adsorption onto the capillary wall. To overcome the limitations of the UV detection method, we incorporated fluorescently labeled phosphatidylcholine into our liposomal formulation which allowed us to distinguish between fluorescently labeled liposomes and other compounds. By utilizing laser-induced fluorescence detection, we proved that liposome adsorption onto the capillary wall was occurring.

The second part is focused on the dynamic and permanent coating of the capillary wall to overcome problems with liposome adsorption. Several dynamic coating approaches were tested for four different polymer coating agents and evaluated based on the suppression of the electroosmotic flow and the coating stability. Linear polyacrylamide was used for permanent coating and its performance was compared with the tested dynamic coatings. This part utilized capillary electrophoresis with laser-induced fluorescence detection to enhance the sensitivity and specificity of liposome detection.

Lastly, liposomes were used as a pseudostationary phase in liposomal electrokinetic chromatography for monitoring drug-lipid interactions. We focused on how varying liposome concentration in the background electrolyte affects the separation kinetics. The presence of liposomes altered the electrophoretic mobility, peak shapes, or both, of several active pharmaceutical ingredients. For instance, uncharged canagliflozin migrated out of the neutral zone in the presence of liposomes and its electrophoretic mobility linearly increased with increasing liposome concentration. Additionally, we used liposomes mimicking natural membrane compositions, prepared from bovine liver or heart tissue extracts. We observed that the interactions also differentiate based on lipid composition with liver extract-based liposomes exhibiting on average stronger effect on the migration of active ingredients. We then examined the effects of temperature and pH on these interactions for nine lipophilic drugs. Increased temperature enhanced the effective mobility of most drugs due to the lower background electrolyte viscosity and increased lipid bilayer fluidity. However, the effect of pH was inconclusive as both, liposome presence and pH changes, influenced drug behavior making the data analysis very complex. Our findings highlight the importance of considering liposome composition, temperature, and pH when studying API-liposome interactions.

# ABSTRAKT (CZ)

První část této disertační práce zkoumá možnosti analýzy a charakterizace liposomů pomocí kapilární elektroforézy. Naším hlavním cílem bylo určit vhodný základní elektrolyt a optimalizovat experimentální podmínky tak, aby se minimalizovala adsorpce liposomů na vnitřní stěnu kapiláry. Abychom překonali omezení UV detekce, přidali jsme do membrány liposomů fluorescenčně značený fosfatidylcholin, což nám umožnilo rozlišit mezi fluorescenčně značenými liposomy a ostatními sloučeninami. Pomocí laserem indukované fluorescenční detekce jsme pak prokázali, že dochází k adsorpci liposomů na stěnu kapiláry.

Druhá část je zaměřena na dynamické a permanentní pokrývání vnitřní stěny kapiláry, vzhledem k problémům s adsorpcí liposomů na její stěnu. Bylo testováno několik přístupů dynamického pokrývání pomocí čtyř různých polymerů, které byly hodnoceny na základě míry potlačení elektroosmotického toku a stability pokrytí. Pro permanentní pokrývání byl použit lineární polyakrylamid a účinnost jeho pokrytí byla porovnána s dynamickým pokrytím. V této části byla pro zvýšení citlivosti a specificity detekce liposomů použita laserem indukovaná fluorescenční detekce .

Nakonec byly liposomy použity jako pseudostacionární fáze v liposomální elektrokinetické chromatografii pro monitorování interakcí mezi léčivy a lipidy. Zaměřili jsme se na to, jak změny koncentrace liposomů v základním elektrolytu ovlivňují kinetiku separace. Přítomnost liposomů měnila buďto elektroforetickou mobilitu, tvary píků, nebo obojí, u několika léčiv. Například neutrální canagliflozin vymigroval z neutrální zóny v přítomnosti liposomů a jeho elektroforetická mobilita se lineárně zvyšovala se zvyšující se koncentrací liposomů. Dále jsme použili liposomy napodobující přirozené složení membrán, připravené z extraktů hovězích jater či srdce. Zjistili jsme, že interakce se liší v závislosti na složení lipidů, přičemž liposomy připravené z extraktů jater vykazovaly v průměru silnější účinek na migraci léčiv. Poté jsme zkoumali vliv teploty a pH na tyto interakce pro devět lipofilních léčiv. Zvýšená teplota zvýšila efektivní mobilitu většiny léčiv díky nižší viskozitě základního elektrolytu a zvýšené fluiditě lipidové dvojvrstvy. Vliv pH byl však nejednoznačný, protože chování léčiv ovlivňovala jak přítomnost liposomů, tak i změny pH, což činilo analýzu dat velmi složitou. Tato část práce poukazuje na důležitost zohlednění několika faktorů, jako složení membrány, teplota a pH, při studiu interakcí farmaceutických látek s liposomy.

**Keywords:** Active pharmaceutical ingredients; Capillary coating; Capillary electrophoresis; Interactions; Laser-induced fluorescence; Liposomal electrokinetic chromatography; Liposomes, Pseudostationary phase

**Klíčová slova:** Farmaceuticky aktivní látky; Interakce; Kapilární elektroforéza; Laserem-indukovaná fluorescence; Liposomální elektrokinetická chromatografie; Liposomy; Pokrývání kapilár, Pseudostacionární fáze

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# LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| 5-FU | 5-fluorouracil |
| API | active pharmaceutical ingredient |
| BBB | blood-brain barrier |
| BGE | background electrolyte |
| CE | capillary electrophoresis |
| CEC | capillary electrochromatography |
| Chol | cholesterol |
| CTAB | hexadecyltrimethylammonium bromide |
| CZE | capillary zone electrophoresis |
| DLS | dynamic light scattering |
| DMPE | dimyristoylphosphatidylethanolamine |
| DPPC | dipalmitoylphosphatidylcholine |
| DPPG | dipalmitoylphosphatidylglycerol |
| DSPC | distearoylphosphatidylcholine |
| DSPG | distearoylphosphatidylglycerol |
| EE | encapsulation efficiency |
| EOF | electroosmotic flow |
| HEPES | 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid |
| LEKC | liposomal electrokinetic chromatography |
| LIF | laser-induced fluorescence |
| LPA | linear polyacrylamide |
| LUVs | large unilamellar vesicles |
| MLVs | multilamellar vesicles |
| MVVs | multivesicular vesicles |
| NBD-PC | phosphatidylcholine with nitrobenzoxadiazol |
| PA | phosphatidic acid |
| PAMPA | parallel artificial membrane permeability assay |
| PC | phosphatidylcholine |
| PDADMAC | polydiallyldimethylammonium chloride |
| PE | phosphatidylethanolamine |
| PEG | polyethylene glycol |
| PG | phosphatidylglycerol |

| | |
|---|---|
| PI | phosphatidylinositol |
| PS | phosphatidylserine |
| PVA | polyvinyl alcohol |
| PVP | polyvinyl pyrrolidone |
| RSD | relative standard deviation |
| SD | standard deviation |
| SPE | solid-phase extraction |
| SUVs | small unilamellar vesicles |
| UV | ultraviolet |

| | |
|---|---|
| $A$ | absorbance |
| $c$ | molar concentration |
| $D$ | distribution coefficient |
| $l$ | length |
| $P$ | partition coefficient |
| pH | decadic logarithm of hydrogen ion activity |
| $S$ | signal |
| $T$ | temperature |
| $t$ | time |
| $\mu$ | electrophoretic mobility |
| $\lambda$ | wavelength |

# 1 INTRODUCTION

## 1.1 Liposomes

Liposomes are small laminar vesicles made up of one or more closed phospholipid bilayers. Each lipid has a hydrophilic head and hydrophobic tail, allowing for the incorporation of both hydrophilic and hydrophobic substances. Hydrophilic ones can be encapsulated within the aqueous core of the liposome, while hydrophobic ones can be incorporated into the lipid bilayer (**Fig. 1**). This makes them a versatile delivery system [1-3]. Since their discovery in the mid-20$^{th}$ century [4,5], liposomes have gained significant attention due to their unique biocompatibility, biodegradability, nontoxicity, and amphiphilic character [6]. These characteristics make them a valuable tool in various fields, particularly in pharmaceuticals, for their controlled transport and protection (either of a drug or of a healthy tissue) [7].

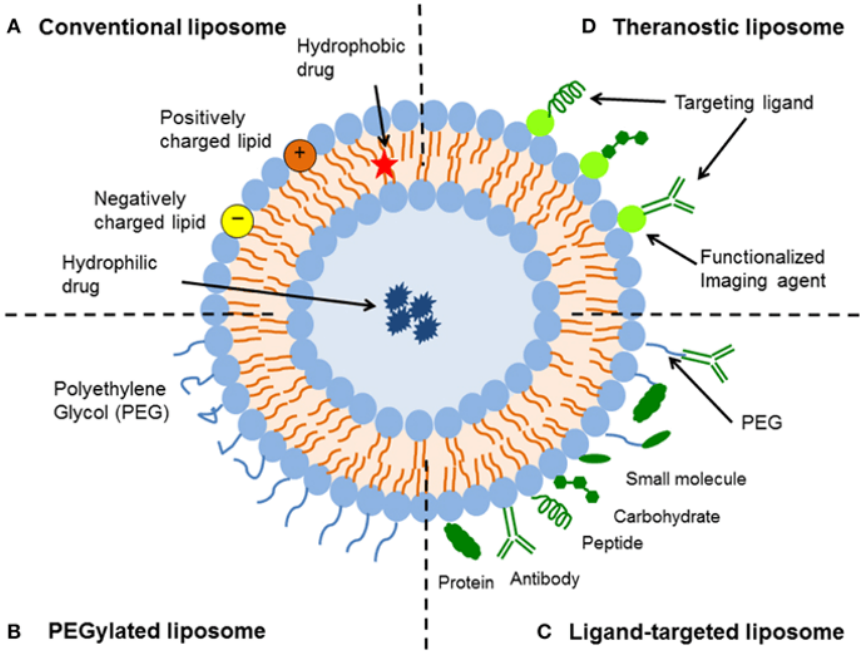



**Fig. 1** – Types of liposomal drug delivery systems [8].

Liposomes have been widely studied and used in various applications. Their ability to encapsulate and deliver drugs has led to their use in numerous pharmaceutical formulations. They are used to improve the therapeutic index of drugs, reduce their toxicity, and enhance their delivery to specific tissues or cells. Their versatility makes them suitable for delivering small molecules, proteins, and nucleic acids [7,9]. Nowadays, liposomes are also used in vaccines, such as hepatitis, malaria, tuberculosis, and more recently, the mRNA COVID-19 vaccines

[10]. Beyond pharmaceuticals, liposomes are used in the food and cosmetic industries to encapsulate and protect active ingredients from degradation or to facilitate deeper skin penetration [11,12].

### 1.1.1 Composition

The key components of liposomes are phospholipids and their overall composition can be adjusted for specific applications (see **Fig. 1**). Commonly used phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA) and phosphatidylinositol (PI) [2]. Incorporating cholesterol (Chol) in liposome formulations can alter membrane fluidity and enhance overall stability [13]. Hydrophilic polymers are added to form sterically stabilized liposomes while targeting ligands such as antibodies or peptides are added to enable the liposomes to bind to specific cells or tissues [8]. Lastly, the core of the liposome or its membrane can be loaded with a variety of therapeutic or diagnostic compounds [14]. Understanding the composition of liposomes is essential for optimizing their performance in drug delivery, diagnostics, and other biomedical applications.

### 1.1.2 Classification

Liposomes are commonly classified based on their size and the number of bilayers (lamellarity) [1-3]. They can be divided into four classes: small unilamellar vesicles (SUVs), which have one phospholipid bilayer and a diameter of 20-100 nm; large unilamellar vesicles (LUVs), which have one phospholipid bilayer and a diameter of 100-1000 nm; multilamellar vesicles (MLVs), which have multiple phospholipid bilayers and a diameter above 500 nm and multivesicular vesicles (MVVs), which contain smaller vesicles within the main vesicle and have a diameter above 1000 nm. Both size and lamellarity have a significant effect on the encapsulation efficiency [15], a value characterizing liposomes based on their transport capacity. Generally, smaller vesicles have higher encapsulation efficiency but may have shorter circulation times due to rapid clearance by the immune system.

Based on their application [2], liposomes can be further divided into four classes: conventional, sterically stabilized, targeted, and stimuli-responsive liposomes. Conventional liposomes are prepared from natural or synthetic phospholipids and are referred to as first-

generation liposomes. Sterically stabilized liposomes with incorporated molecules such as polyethylene glycol (PEG) or polyvinyl alcohol (PVA) within the bilayer are used to extend the circulation time in the body, because their recognition and clearance by the immune system is reduced. Both passive and active targeted liposomes increase selective interactions with cells by incorporating targeting ligands, which enhances delivery to specific sites. Stimuli-responsive liposomes show rapid drug release in response to biochemical or physicochemical stimuli such as redox potential, enzyme concentration, pH, or temperature [16-18].

Liposomes can also be classified based on their charge [2,19], which influences their behavior in biological systems and their stability, into three classes: cationic, neutral, and anionic. Cationic liposomes carry a positive charge and they are often used in gene therapy because they can form complexes through electrostatic interactions with negatively charged molecules, such as nucleic acids. Neutral liposomes are typically composed of neutral (zwitterionic) phospholipids and they tend to have longer circulation times in the bloodstream because they have a lower tendency to interact with cells and proteins. Anionic liposomes carry a negative charge and they show higher clearance from the body due to the lower in-body stability. They are often used for transdermal application as they have enhanced penetration through the skin [20].

### 1.1.3 Preparation

The preparation of liposomes involves several different methods, each used to achieve specific characteristics such as size, lamellarity, and encapsulation efficiency [6]. The selection of an appropriate preparation method is crucial because it directly affects the performance and application of the liposomal formulation [21].

The lipid film hydration method also known as Bangham method (**Fig. 2**) is the most commonly used method for liposome preparation [22-24]. It involves dissolving the lipids and a hydrophobic drug in an organic solvent in a round-bottom flask. By evaporating the organic solvent using rotary evaporation, a thin film of lipids is created on the inner wall of the flask. This film is then hydrated with an aqueous solution, which may contain a hydrophilic drug. Intensive stirring or shaking then produces MLVs. Unilamellar vesicles of uniform size may be obtained from MLVs, either by sonication or extrusion. During sonication, smaller vesicles are created using ultrasound with a sonication probe or in a sonication bath. During extrusion, the sample is forced through a polycarbonate membrane, forming liposomes with a diameter similar

to the specific pore size of the membrane. The advantage of extrusion is its good reproducibility; however, it is a more time-consuming method.

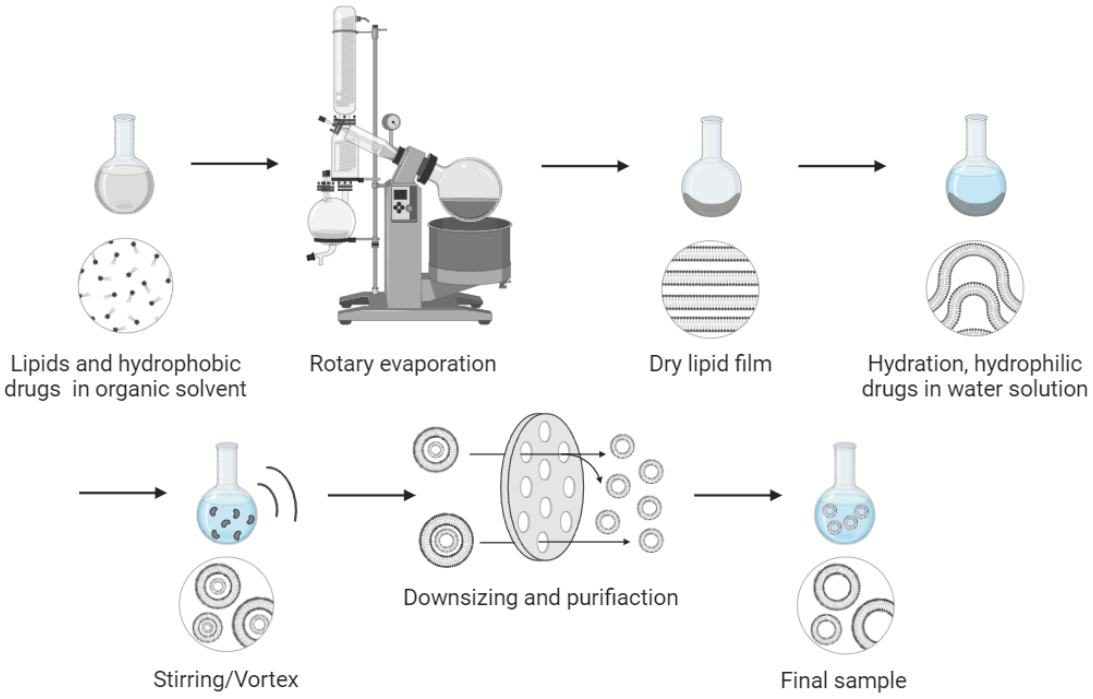



**Fig. 2** – Liposome preparation using lipid film hydration followed by sample treatment. Adapted from [25].

Other methods for liposome preparation include ethanol/ether injection method [2,3, 26], which is based on the injection of a lipid solution, which is dissolved in a water-miscible organic solvent (ethanol or ether), into a stirred aqueous phase. This leads to the spontaneous formation of SUV liposomes and the organic solvent is then removed either by dialysis, ultrafiltration, or evaporation [27].

The emulsification method (also called reverse-phase evaporation method) [28] involves dissolving lipids in an organic solvent and then mixing them with an aqueous phase to form water-in-oil emulsions. The organic solvent is then gradually evaporated under reduced pressure resulting in the formation of LUVs liposomes. This method is used for encapsulating hydrophilic drugs with higher encapsulation efficiency compared to injection methods [29].

During microfluidic channel method [30], the lipids dissolved in an organic solvent, such as ethanol or isopropanol, are continuously mixed with an aqueous solution within the microfluidic channel leading to liposome formation. The control over the flow rates and mixing conditions allows for the formation of liposomes with a high degree of uniformity and

reproducibility. Additionally, this method can be used for the large-scale production of liposomes, making it a promising approach for industrial applications [31].

### 1.1.4 Characterization of liposomes

To make sure liposomes are stable and effective, it is important to characterize them using a variety of methods. Thorough characterization ensures that the liposomes will perform as intended for their specific applications [32]. Dynamic light scattering (DLS) is used to determine the size distribution and the polydispersity of the sample [33]. Zeta potential measurement is used to obtain the surface charge, which affects not only the sample stability but also the interactions with biological membranes. Accurate zeta potential ensures that the liposomes will repel each other and remain in suspension without aggregating [34,35]. Transmission electron microscopy provides detailed images of sample morphology and its lamellarity [36]. The obtained information about their size, shape, and number of bilayers helps in evaluating their potential applications. Furthermore, analytical techniques, such as high-performance liquid chromatography [37], capillary electrophoresis (CE) [38], and spectrophotometry [39], are used to quantify the amount of encapsulated drug and to monitor its release. Ensuring that liposomes contain the proper drug dosage and that it is released at the desired rate is crucial for the safety of liposomal drug formulations.

Encapsulation efficiency (EE) is a key parameter in the liposome formulation; it represents the percentage of a compound that is encapsulated within the liposome relative to the total amount that was added during preparation [2,40]. High encapsulation efficiency indicates effective loading of the compound, leading to better therapeutic efficiency and reduced losses of the compound. To remove the free compound surrounding the liposome, techniques such as centrifugation, extraction, or filtration can be used, leading to supernatant, extract, or filtrate containing the free compound [3,41]. This ensures that only the encapsulated drug is delivered within the body and unnecessary exposure to the free compound is eliminated. Encapsulation efficiency is calculated based on **Equation 1** as follows:

$$EE = \left(\frac{c_{total} - c_{free}}{c_{total}}\right) \cdot 100\ \% \qquad (1),$$

where $EE$ is the encapsulation efficiency, $c_{\text{total}}$ is the amount of added drug, and $c_{\text{free}}$ is the amount of free drug determined by an analytical technique.

## 1.2 Liposomes and capillary electrophoresis

Capillary electrophoresis is a powerful tool in the study of liposomes, providing insights into their characteristics, such as size and charge, encapsulation efficiency, permeability of the phospholipid membrane, and interactions with other compounds through capillary electrochromatography (CEC) or liposomal electrokinetic chromatography (LEKC).

### 1.2.1 Size and charge

One of the less common uses is the determination of liposome size and charge by CE. Both those parameters affect the behavior of liposomes in biological systems. Compounds are separated based on their electrophoretic mobility in CE, which is influenced by both, size and charge [42].

Wiedmer et al. [43] studied the effect of increasing the amount of anionic lipid in the liposome sample on electrophoretic mobility. They proved that as the amount of negatively charged lipids increased, the negative surface charge also increased. Several other groups [44-46] have also calculated the liposome charge from measured electrophoretic mobility and data obtained from DLS. Elsewhere, electrophoretic mobility measurement was used to determine zeta potentials, which decreased with increasing amount of anionic lipids [47,48].

Radko et al. [49] found that liposomes prepared from PC/PG/Chol with similar charges but different sizes have different mobilities in BGEs of low ionic strength.

In recent years, Taylor dispersion analysis performed in the capillary gained interest as a method for size distribution determination [50,51]. It is based on measuring liposome peak dispersion due to the parabolic flow profile in the capillary.

### 1.2.2 Membrane permeability

The transport of pharmaceutical compounds is a critical aspect of drug development, and one of the most common mechanisms is passive diffusion. Passive diffusion refers to the movement of drugs across biological membranes driven by concentration gradients, and it is particularly important for orally administered drugs [52,53]. When evaluating the suitability of active pharmaceutical ingredients (APIs) for passive diffusion, several characteristics come into play, and they can be categorized into four different classes based on their permeability and

solubility (**Fig. 3**): from insoluble and non-permeable (class IV) to soluble and permeable (class I) [54].

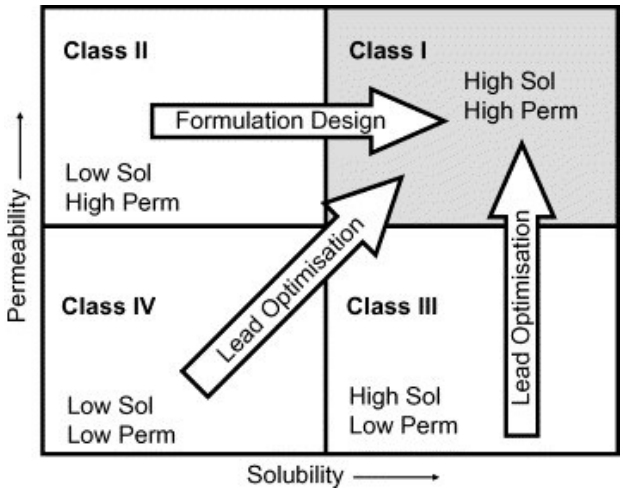



**Fig. 3** – Biopharmaceutical classification system [55].

Lipophilic compounds have an affinity for lipids and are more likely to pass through cell membranes. Lipophilicity is often quantified using parameters such as partition coefficient (log P) and distribution coefficient (log D), which provide information about a compound's hydrophobicity [56]. While lipophilicity is essential, drugs should also be sufficiently soluble in water to be absorbed into the bloodstream and distributed throughout the body. Solubility is a key factor in oral drug absorption [55]. Membrane permeability is the ability of a drug to cross cell membranes. Lipophilic and soluble compounds are more likely to be membrane-permeable. This property is crucial for drugs to reach their target sites within the body [57]. The evaluation of permeability through the membrane can be done by following methods: *in silico* methods (computational models and simulations) [58], *in vitro* methods (artificial membranes; the common technique is parallel artificial membrane permeability assay, PAMPA) [59], cell-based methods (live cells such as Caco-2) [60] and non-cell-based methods where capillary electrophoresis comes into play [3]. CE offers a valuable alternative to studying the permeability of the phospholipid bilayer, which is essential for monitoring of unwanted leakage or for observing how the compound is released under various conditions. Factors like lipid composition, temperature, pH, and ionic strength influence permeability. Maximum permeability was observed at the phase transition temperature; on the other hand, presence of cholesterol in the liposome sample reduces it [61,62].

Tsukagoshi et al. [63] studied the release of Eosin Y dye from liposomes with increasing storage temperature by CE with chemiluminescence detection. They found that temperature

above phase transition temperature increased the dye release. In their further studies [64], they revealed that a lower concentration of the buffer inside the cavity compared to the surrounding buffer increased dye leakage likely due to the osmotic shrinkage.

Weiss et al. [65] quantified the leakage of liposome-encapsulated dye by chip electrophoresis. The leakage was induced by viruses or by virus-derived peptides by reacting with receptors on the liposomal membrane.

### 1.2.3 Liposomal electrokinetic chromatography

CEC or LEKC (**Fig. 4**) offer insights into how drugs interact with different carriers and membranes, they are also used to determine the liposome/water partition coefficients. CEC combines elements of capillary electrophoresis and chromatography. It involves the use of liposomes as stationary phases attached to the inner wall of the capillary to facilitate chromatographic separation. It combines electrophoretic mobility and chromatographic interactions with the liposomal phase and EOF for separation. LEKC is similar, however, the liposomes are freely suspended in the background electrolyte and they are used as a pseudostationary phase [3].

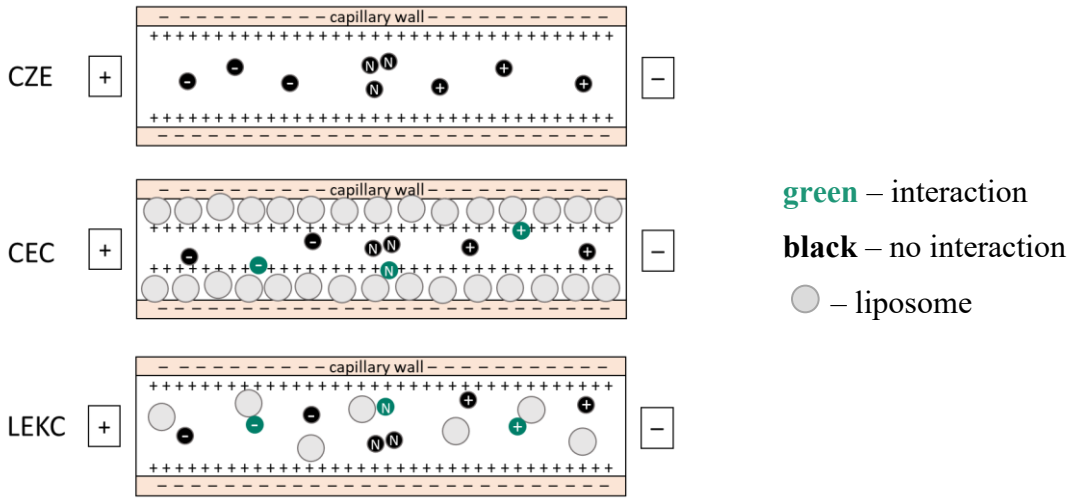



**Fig. 4** – Different modes in capillary electrophoresis. CZE – capillary zone electrophoresis without liposomes, CEC – capillary electrochromatography with liposomal stationary phase attached to inner capillary wall, LECK – liposomal electrokinetic chromatography with liposomal pseudostationary phase added to background electrolyte.

In both cases, the interaction between APIs and liposomes can influence the kinetics of analytical separations and it can manifest in various ways, including changes in mobility and/or peak shapes. Despite several advantages, problems with preparing liposomes with consistent properties and the complexity of data interpretation need to be taken into consideration.

Wiedmer et al. [66] also studied the interactions of six steroid hormones with liposomes with increasing amounts of cholesterol in the liposomal formulation. Relative migration times showed that increasing cholesterol content affected steroid partitioning into membranes based on their hydrophobicity. For less hydrophobic compounds, there was a decrease in migration times, indicating lower partitioning of these steroids into membranes with high cholesterol content. For more hydrophobic compounds, partitioning initially increased and then decreased. Lastly, β-estradiol as the only compound with aromatic ring and with two hydroxyl groups, showed no changes in mobilities at first, but with higher cholesterol content, there was a significant decrease in the interaction with liposomes.

Jiang et al. [67] focused on the determination of the lipid-water partition coefficients ($K_{L/W}$) of 9 neutral and 23 charged drugs. They used DSPC/Chol (LUVs) and soybean lecithin/Chol (MLVs) liposomes and they observed a good linear relationship with $R^2 = 0.89$ between individual log $K_{L/W}$ determined by LEKC using these two types of liposomes. Moreover, the results indicated that DSPC/Chol as LUVs liposomes better simulated cell membranes than soybean lecithin/Chol liposomes as it showed a better linear relationship with software prediction and Caco-2 cell model.

Godyń et al. [68] presented a novel method for screening the passage of compounds through the blood-brain barrier (BBB) using CEC. They used liposomes prepared from POPC/PS in a molar ratio of 80:20 or liposomes prepared from porcine brain extract for capillary coating and determined retention factors (log k) for 25 drugs. They compared the data obtained from CEC with log $P_e$ values obtained by PAMPA-BBB and with *in vivo* log BB data found in the literature by calculating correlation coefficients.

The study by Amézqueta et al. [69] evaluated the use of lecithins as a potential pseudostationary phase in EKC or mobile phase in liquid chromatography through the solvation parameter model. They prepared either microemulsion or liposomes from ready-to-use lecithin and tested their effectiveness in these systems. They found that lecithin-based systems offer a robust and practical alternative for predicting the skin partitioning of neutral solutes, particularly with the lecithin microemulsion EKC method.

## 1.3 Capillary coating

As mentioned previously, a significant challenge is liposome's tendency to be adsorbed onto the surface of the capillary, which leads to peak broadening, tailing, and thus to poor reproducibility. To prevent liposome adsorption, which in turn brings improved separation efficiency, various strategies for coating the inner surface can be used. Three common types of capillary coatings are dynamic, permanent, and semi-permanent coating [70,71]. At the same time, the coating of the capillary (**Fig. 5**) leads to modification of the EOF. The most important parameters in the capillary coating are its reproducibility, homogeneity, and stability.

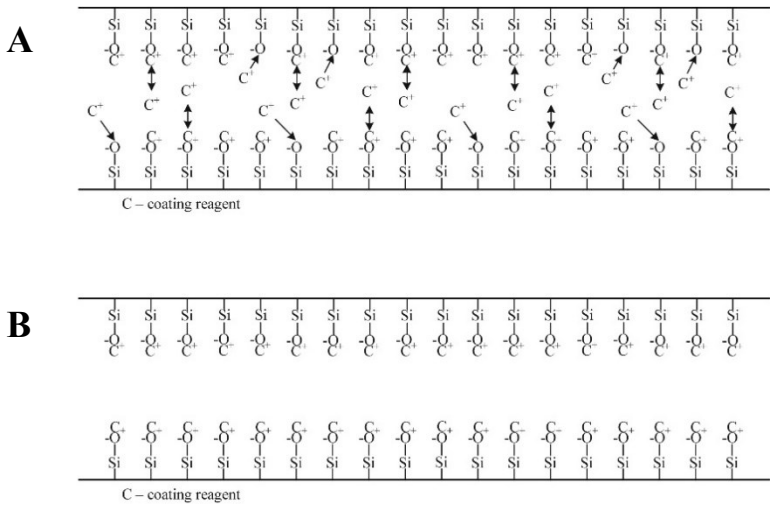



**Fig. 5** – Dynamic (A) and permanent (B) capillary coatings [70]

### 1.3.1 Dynamic coating

Dynamic coating is employed in the capillary electrophoresis to modify the EOF. This method involves flushing the capillary with a coating agent, which can also be added to the BGE in small amounts to prevent gradual washing out. The coating agent reversibly adsorbs onto the inner surface, predominantly through secondary adsorption interactions with the silanol groups present on the capillary [71].

Typically, polymers are used for dynamic coating due to their versatile properties. These polymers can be either neutral or charged, each serving a specific purpose in modulating the EOF [72]. Neutral polymers, such as PVA, PEG, or polyvinyl pyrrolidone (PVP), are effective in suppressing the EOF. In contrast, charged polymers are used to manipulate the EOF direction

and velocity. Positively charged polymers can reverse the EOF direction and negatively charged polymers can accelerate the EOF.

Additionally, dynamic coating can be achieved by using cationic surfactants. Surfactants, such as cetyltrimethylammonium bromide (CTAB) or didodecyl-dimethylammonium bromide, are effective in reversing the EOF direction. These surfactants form a positively charged double layer on the capillary surface, which inverts the charge of the capillary wall. Anionic analytes then migrate in the opposite order, in the same direction with the EOF, and thus the total analysis time is shortened [73,74]. To ensure proper separation, it is necessary to reverse the polarity of the applied voltage.

### 1.3.2 Permanent coating

Permanent coating involves the covalent attachment of a coating agent to the capillary wall. This method provides consistent performance and generally higher stability compared to dynamic coating. On the other hand, the process is more time-consuming and typically consists of three main steps: the surface of the capillary is first activated, then the silanization occurs and finally the coating agent is covalently bonded [*72*,75,76].

The capillary surface is first treated with strong acid (such as hydrochloric acid) or base (such as sodium hydroxide) to introduce reactive groups. This step is crucial for ensuring effective silanization and subsequent coating. Then the capillary is flushed with deionized water and completely dried, either by using an inert gas or in an oven. In the next step, the modified capillary surface undergoes silanization. It involves a reaction between activated silanol groups and the silanization agent, forming a stable layer that is ready to react with the coating agent. Finally, the coating agent is covalently bonded to the silanized surface. This ensures the permanent attachment of the coating, providing long-term stability and reproducibility [72,77].

# 2 EXPERIMENTAL

## 2.1 Liposomes

In this work, various types of liposomes have been used, differing in their composition (**Fig. 6**) and/or hydration buffer. These liposomes were prepared by the research group of prof. Ing. František Štěpánek, Ph.D., from the Institute of Chemical Engineering at the University of Chemistry and Technology. All liposomes used in this work were prepared by lipid hydration method, briefly: individual lipids were dissolved in the chloroform:methanol mixture (2:1) in a round flask, the organic solvent was then evaporated in a rotary evaporator at 60 °C while gradually lowering pressure from 350 mbar to approximately 120 mbar, formed lipid film was kept overnight under vacuum, lipid suspension was obtained after rehydration of the film with buffer and stirring it by vortex shaker, this suspension was then transferred to extrusion device heated to 69 °C and extruded through a membrane with a defined pore size 21 times. Initially, the liposome samples were prepared by Ing. Martin Balouch, Ph.D., and later on, by myself.

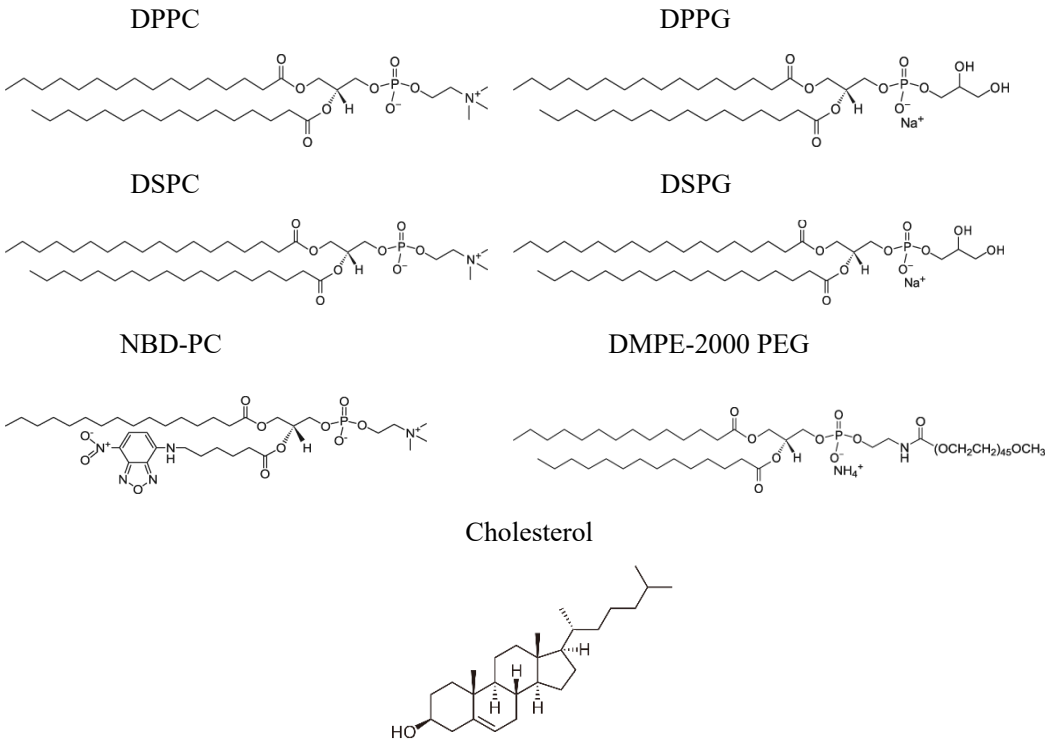



**Fig. 6** – Structures of used lipids and cholesterol. DPPC – dipalmitoylphosphatidylcholine, DPPG – dipalmitoylphosphatidylglycerol, DSPC – distearoylphosphatidylcholine, DSPG – distearoylphosphatidylglycerol, NBD-PC - fluorescently labeled phosphatidylcholine with nitrobenzoxadiazole, DMPE-2000 PEG –PEGylated dimyristoylphosphatidylethanolamine.

- *DPPC:DPPG*

Initial experiments were done with liposomes prepared from dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) lipids of a total concentration of 5 mg/ml in 75:25 molar ratio. Lipids were hydrated in 10 mmol/l sodium phosphate buffer at pH 7.10 and extruded through a 100 nm membrane.

- *DSPC:DSPG:DMPE-2000PEG*

More stable liposomes were prepared from distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), and polyethylenglycolated (PEG) dimyristoyl-phosphatidylethanolamine (DMPE) lipids of a total concentration 5 mg/ml in 75:22:3 molar ratio. Lipids were hydrated in 1 mmol/l calcium gluconate with 10% sucrose and extruded through a 100 nm membrane.

- *DSPC:DSPG:DMPE-2000PEG:NBD-PC*

Liposomes with fluorescent tag were prepared for measurements with laser-induced fluorescence detection from DSPC, DSPG, DMPE-2000 PEG, and from fluorescently labeled PC (**Fig. 7**) with nitrobenzoxadiazole (NBD) of a total concentration of lipids 5 mg/ml in 75:22:3:0.5 molar ratio. Lipids were first hydrated in 1 mmol/l calcium gluconate with 10% sucrose, later on in 10 mmol/l sodium phosphate buffer at pH 7.10, and extruded through a 100 nm membrane.

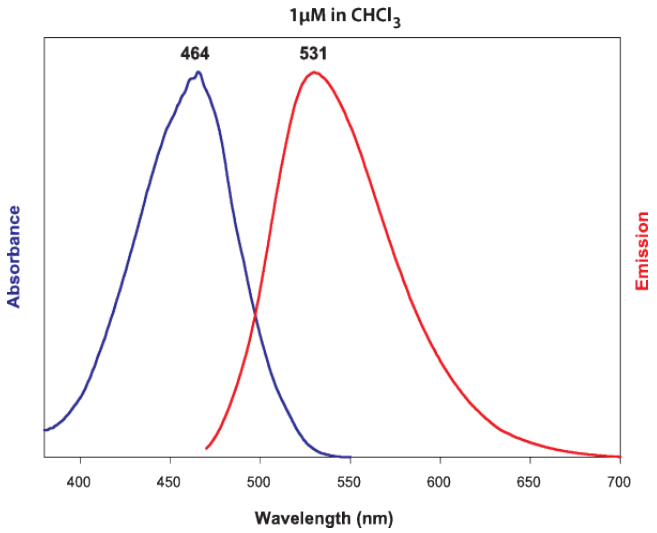



**Fig. 7** – Absorbance and emission spectrum of NBD-PC lipid [78]

- *DPPC:DPPG:Chol*

Four liposomes of various amounts of cholesterol were prepared from DPPC, DPPG, and Chol in molar ratios of 75:25:0, 65:25:10, 55:25:20, and 45:25:30, respectively. Lipids were hydrated in 10 mmol/l sodium phosphate buffer at pH 7.10 and extruded through a 400 nm membrane.

- *Tissue extract liposomes*

Liposomes from the bovine liver or bovine heart extracts were prepared so that the total lipid concentration was 5 mg/ml. Lipids were hydrated in 10 mmol/l sodium phosphate buffer at pH 7.10 and extruded through a 400 nm membrane. Liver polar lipid extract (bovine) is composed of various lipids, including PC (42%), PE (26%), PI (9%), lyso PI (1%), Chol (5%) and other neutral lipids (17%). Heart polar lipid extract (bovine) is composed of various lipids, including PC (8.6%), PE (13.6%), PI (1.0%), PA (0.6%), cardiolipin (1.7%), neutral lipids (57.7%) and also 16.8% of the unknown. Both extracts [78] contain negatively charged phospholipids (PI, PA, and cardiolipin), which give them a negative charge.

## 2.2 EOF mobility measurements

### 2.2.1 Dynamically coated capillaries

In dynamically coated capillaries, the EOF mobility was determined by using thiourea as the EOF marker based on **Equation 2**:

$$\mu_{EOF} = \frac{l_t \, l_d}{U \, t_{EOF}} \qquad (2),$$

where $\mu_{EOF}$ is the mobility of the electroosmotic flow, $l_t$ is the total length of the capillary, $l_d$ is the length to the detector, $U$ is the applied voltage, $t_{EOF}$ is the migration time of the EOF marker

### 2.2.2 Permanently coated capillaries

In permanently coated capillaries, the method by Williams and Vigh [79] was employed to determine the EOF mobility. It involved injecting the first zone of thiourea for 5 seconds by 5 kPa, then the zone was mobilized for 60 seconds by BGE at 10 kPa, the same procedure was applied for the second zone of thiourea, then the voltage of 20 kV was applied for 120 seconds, and finally, the third zone of thiourea was injected for 5 seconds by 5 kPa. All zones were then

moved to the detector by BGE at a pressure of 10 kPa. The mobility was calculated based on **Equation 3**:

$$\mu_{EOF} = \frac{(t_3 - 2t_2 + t_1)\, l_t\, l_d}{t_U\, U\, t_3} \qquad (3),$$

where $\mu_{EOF}$ is the mobility of the electroosmotic flow, $t_1$, $t_2$ and $t_3$ are the migration times of individual thiourea zones, $l_t$ is the total length of the capillary, $l_d$ is the length to the detector, $U$ is the applied voltage, $t_U$ is the time of applied voltage

## 2.3 Early-stage separation monitoring

We used the following method for studying the separation in its earlier stages (**Fig. 8**). The sample is hydrodynamically injected into the capillary, moved by pressure approximately half the length of the capillary, voltage is applied for a specified time interval, and then the zones present in the capillary are moved by pressure to the detector. By changing the duration of the voltage application, we can observe the separation of zones at different stages of the process.

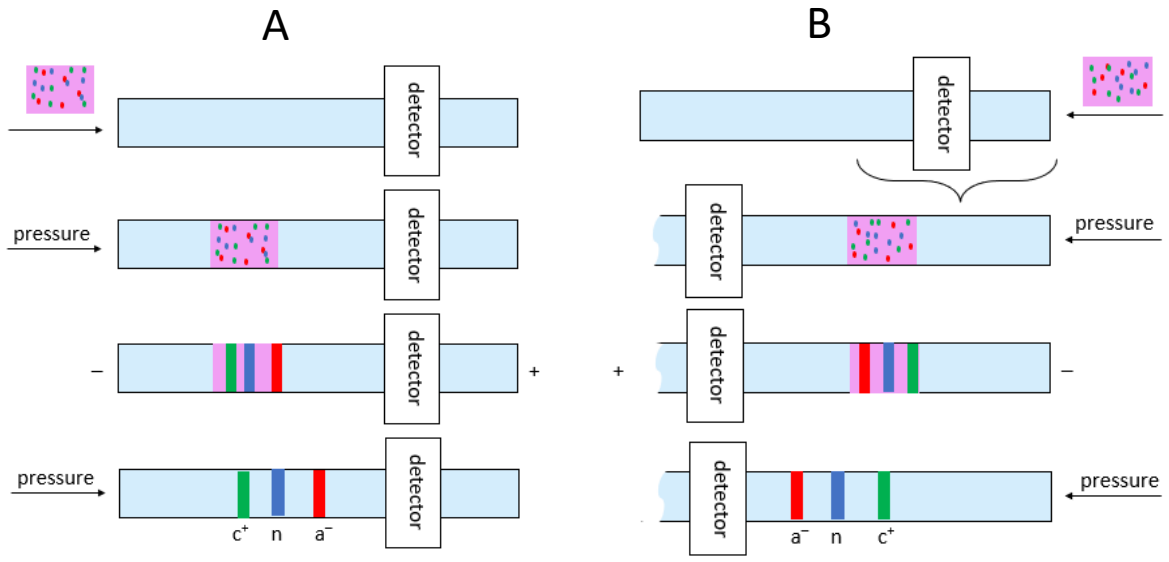



**Fig. 8** – Early-stage separation monitoring. Injection at the long end (A) and short end (B) of the capillary together with migration orders of individual zones based on the charge of the analytes ($c^+$ – cations, n – neutral compounds, $a^-$ – anions).

# 3 RESULTS AND DISCUSSION

## 3.1 Liposome analysis and characterization

Prior to performing liposome analysis by capillary electrophoresis, it was necessary to select a suitable background electrolyte and optimal experimental conditions. The selection of an appropriate BGE is essential, as it significantly affects the analysis outcome. Based on our prior experience, we utilized 10 mmol/l sodium phosphate buffer at pH 7.10 as the initial BGE. This buffer provides a neutral pH environment beneficial for liposome stability and it closely resembles physiological pH conditions, which is crucial for ensuring that the liposomes behave similarly to how they would in a biological system.

We analyzed a liposome sample prepared from DPPC and DPPG lipids mixed at a molar ratio of 75:25. This specific composition was selected to achieve a balance between stability and charge. The presence of DPPG gives a negative charge to the liposomes, which is beneficial for reducing their adsorption onto the negatively charged capillary wall, a common issue that can lead to severe band broadening, loss of sensitivity and selectivity, resulting in inaccurate analysis. A higher concentration of DPPG could lead to excessively high zeta potential, resulting in the instability of the sample due to the electrostatic repulsion among the lipids within the liposomal bilayer.

The initial UV measurement involved hydrodynamically injecting DPPC:DPPG liposomes with 0.1 mg/ml thiourea, which was used as an EOF marker, at 5 kPa for 5 seconds, with an applied voltage of 30 kV during the separation. The results (**Fig. 9**) showed a distinct peak of thiourea and a small, broad peak, likely corresponding to liposomes. For this reason, this peak is labeled as ‘L?’ to indicate uncertainty regarding its identification as the liposome peak. This peak increased with each measurement, which suggested that, despite using negatively charged liposomes, the adsorption onto the inner capillary wall was occurring. To eliminate this, we introduced a flushing step with 1 mol/l sodium hydroxide for 2 minutes, followed by deionized water for another 2 minutes before each measurement. The sodium hydroxide solution effectively removed adsorbed liposomes or their fragments from the capillary by disrupting lipid layers and the deionized water ensured the removal of residual NaOH that could potentially damage liposomes during subsequent runs. Although this flushing step was effective for cleaning the capillary between runs, it did not prevent the liposome-capillary interactions causing the peak broadening.

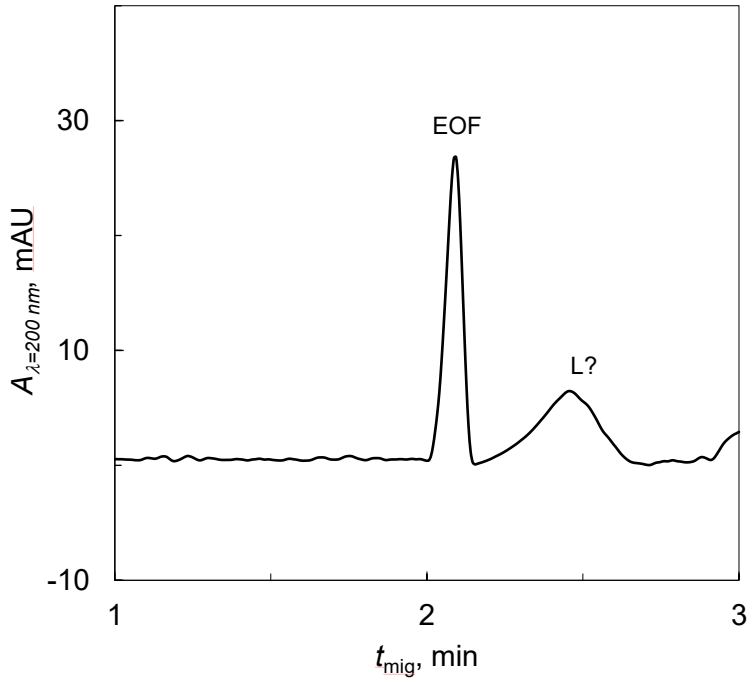



**Fig. 9** – Electropherogram of the DPPC:DPPG liposome with 0.1 mg/ml thiourea; BGE: 10 mmol/l sodium phosphate buffer at pH 7.10. Capillary: 50 µm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec, applied voltage 30 kV; (EOF) thiourea, (L?) DPPC:DPPG liposome.

Subsequently, we tested several BGEs aiming to minimize the liposome adsorption onto the capillary. The tested BGEs were chosen based on their properties or previous literature reports:

- 10 mM sodium phosphate buffer at pH 7.10;
- 25 mM sodium phosphate buffer at pH 7.10;
- 25 mM sodium phosphate buffer with 10% (*w/v*) sucrose at pH 7.10;
- 5 mM HEPES + 5 mM Tris at pH 8.00;
- 10 mM sodium formate buffer at pH 4.10;
- 1 mM calcium gluconate with 10% (*w/v*) sucrose;
- 10 mM borate buffer at pH 9.10;
- 10 mM borate buffer with 10% (*w/v*) sucrose at pH 9.10.

Initially, we increased the ionic strength of the phosphate buffer by using higher concentration of buffer constituents while maintaining the buffer pH. In principle, the higher ionic strength can reduce adsorption by reducing the electrostatic interactions between the capillary wall and the liposomes. Additionally, we explored the effects of adding 10% sucrose to the 25 mM

sodium phosphate buffer as sucrose has been reported to increase liposome stability by providing an osmotic barrier, which could potentially reduce the adsorption onto the capillary wall. We also tested the solution of 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES) with Tris at pH 8.0. HEPES, being a larger anion than phosphate, better corresponds with the electrophoretic mobility of the liposomes, which could reduce the possible adverse effect of electromigration dispersion. Furthermore, we used formate buffer at pH 4.10 to determine whether a lower pH might reduce the charge on both the capillary surface and the liposomes, leading to decreased adsorption. Based on the work of Franzen et al. [50], we also tested the solution of calcium gluconate with sucrose, assuming that calcium ions might form a barrier between liposomes and the capillary wall. Finally, we tested a borate buffer which is commonly used in CE experiments, with and without the addition of sucrose, to find whether the increased viscosity of this BGE might further reduce the liposome adsorption.

Among these, we obtained the most promising results with 25 mmol/l sodium phosphate buffer containing 10% (*w/v*) sucrose. The electropherogram (**Fig. 10**) showed not only the EOF peak, but also a narrow peak of negatively charged liposomes, suggesting that the issue with the adsorption had been reduced.

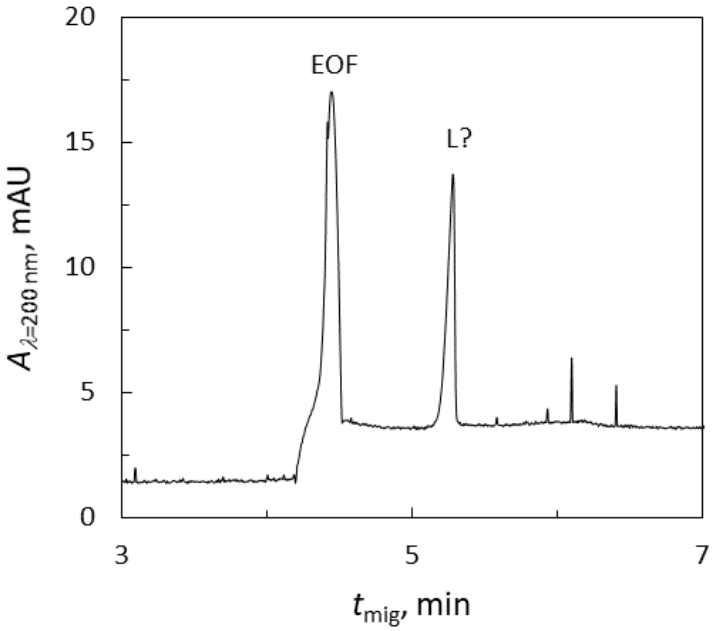



**Fig. 10** – Electropherogram of the DPPC:DPPG liposome with 0.1 mg/ml thiourea; BGE: 25 mmol/l sodium phosphate buffer with 10% (*w/v*) sucrose at pH 7.10. Capillary: 50 μm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec, applied voltage 30 kV; (EOF) thiourea, (L?) DPPC:DPPG liposome.

Usually, CE electropherograms provide only limited picture of the processes occurring within the capillary as they depict the zones at the moment when they pass through the detector. Band-broadening processes taking place during the separation, such as adsorption to the capillary wall or electromigration dispersion, can lead to severe deformation or even complete loss of analyte zone signals. To study the early stages of separation when the effect of band-broadening processes is less pronounced, we employed a method described in **Chapter 2.3**. Briefly, the sample was hydrodynamically injected into the capillary and moved approximately halfway through the capillary length by applying pressure. Voltage was then applied for a short time period and finally, the partially separated zones were moved by pressure to the detector. This method allowed us to observe individual zones at different stages of the run and gain insights into the separation dynamics.

Using this approach, we observed the peaks of thiourea and liposomes (**Fig. 11**) after applying the voltage of 30 kV for different times, ranging from 15 to 60 seconds. Ideally, as the voltage application time increases, the peaks should maintain the same size while their resolution should differ. Despite significant differences in voltage application times, the peak resolution remained practically the same while the peak of the liposome unexpectedly grew, suggesting underlying issues with the separation. To rule out the possibility that this was caused by the presence of sucrose in the BGE, we repeated the same experiment in sodium phosphate buffer without the sucrose, but we observed the same behavior. One possible explanation for this higher peak is that liposomes could have been temporarily adsorbed onto the capillary wall during the pressure mobilization, leading to their unchanged mobility and increased size. This behavior is similar to chromatographic separation, where different components are separated based on their interactions with the stationary phase (in this case, the capillary wall). Given these preliminary experiments, it may be worth questioning whether the observed peaks truly belonged to liposomes or not.

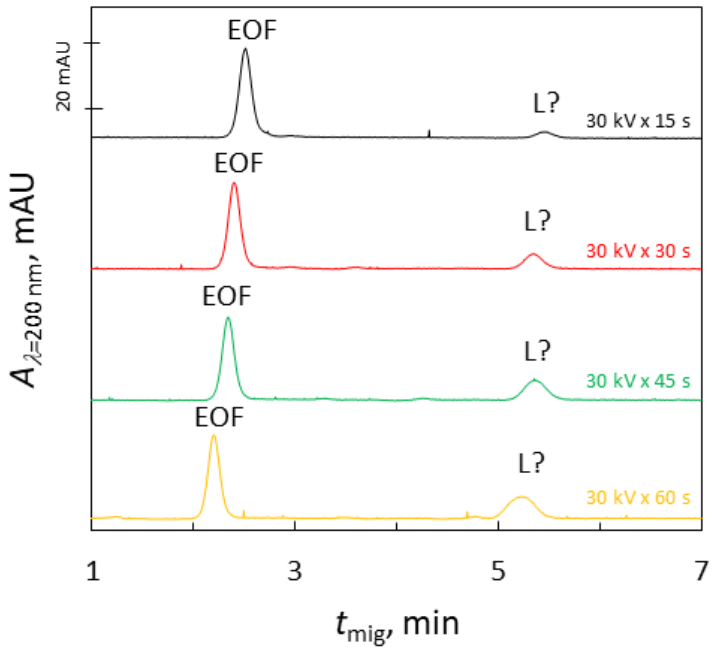



**Fig. 11** – Electropherogram of the DPPC: DPPG liposome with 0.1 mg/ml thiourea with increasing time of applied voltage;
BGE: 25 mmol/l sodium phosphate buffer with 10% (*w/v*) sucrose at pH 7.10. Capillary: 50 µm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec, pressure 10 kPa for 170 sec, applied voltage 30 kV for different time periods and final pressure 10 kPa; (EOF) thiourea, (L?) DPPC:DPPG liposome.

Given these unexpected results, we decided to seek other CE conditions that would provide more reliable results. In the conventional CE setup, the EOF is directed towards the cathode and negatively charged molecules migrate against the EOF towards the anode. If these compounds exhibit relatively low effective mobility, the EOF carries them towards the cathode but their anodic mobility retards them strongly, resulting in a very long migration time. On the other hand, if their mobility is high, they may migrate out of the capillary without passing through the detector. Therefore, to separate negatively charged molecules, the direction of the EOF is often reversed.

We used cationic surfactant CTAB to reverse the EOF. To achieve proper separation, we reversed the polarity of the applied voltage. First, we examined the effect of CTAB concentration on the EOF mobility (**Fig. 12**) that was calculated based on Equation 3. We observed, that with increasing CTAB concentration in the BGE, the originally cathodic EOF mobility reversed to anodic between the concentrations of 0.1 to 0.2 mmol/l of CTAB. The maximum anodic EOF mobility value was at 0.6 mmol/l CTAB, where the capillary was fully

coated with this surfactant. With further increase of CTAB concentration, the mobility slightly decreased, which might be due to increasing ionic strength of the BGE.

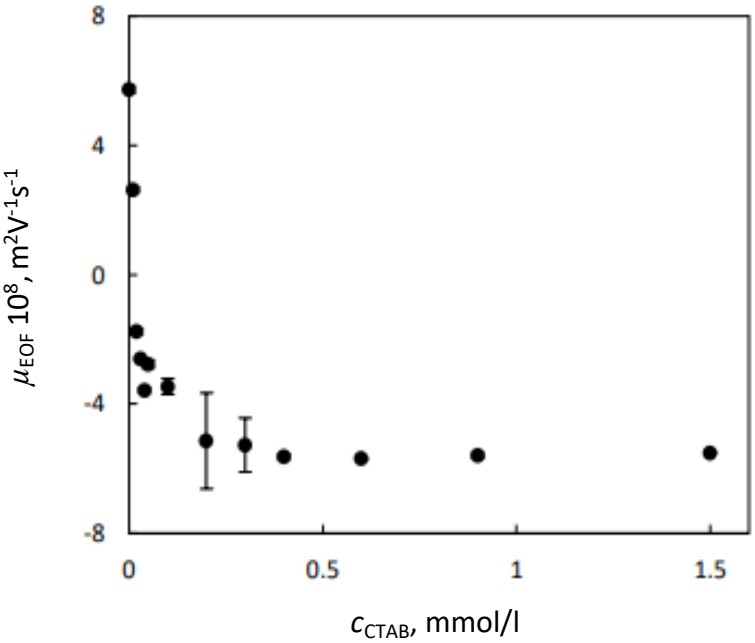



**Fig. 12** – The effect of CTAB concentration on the EOF mobility;
BGE: 10 mmol/l borate buffer at pH 9.10 with increasing amount of CTAB. Capillary: 50 µm ID, total length 50.0 cm/41.5 cm effective length; measurement done by a method described in **Chapter 2.2**

We then analyzed DPPC:DPPG liposomes in 10 mmol/l borate buffer at pH 9.10, both without CTAB and with CTAB at 0.2 mmol/l concentration (**Fig. 13**). The borate buffer alone did not provide efficient separation as once again; a very broad peak was present. Moreover, this peak had an additional sharper peak at the end (**Fig. 13A**). The liposome formulation may have contained varying fractions, leading to a different migration behavior. Larger liposomes could produce a broader peak, while smaller liposomes might generate a narrower one. The addition of CTAB significantly enhanced the analysis of liposomes. With the reversed polarity and corresponding reversed EOF, the liposome peak appeared before the EOF peak and it was very narrow and high (**Fig. 13B**).

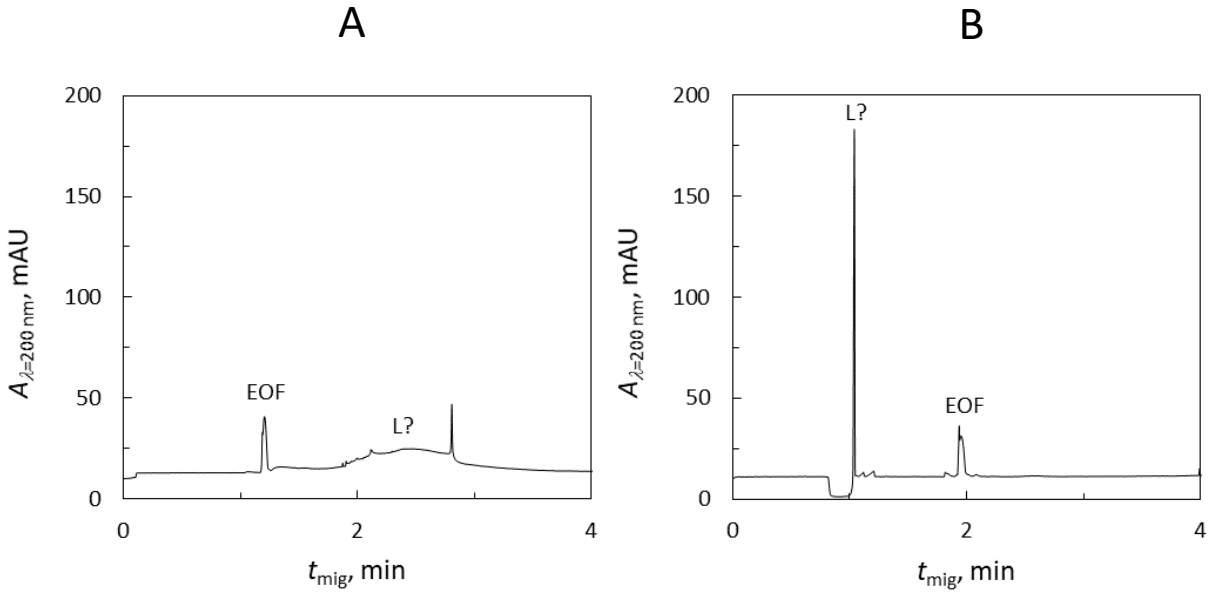



**Fig. 13** – Electropherogram of the DPPC:DPPG liposome with 0.1 mg/ml thiourea; BGE: 10 mmol/l borate buffer at pH 9.10 without (A) and with (B) 0.2 mmol/l CTAB. Capillary: 50 μm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec; applied voltage 30 kV (A) or –30 kV (B); (EOF) thiourea, (L?) DPPC:DPPG liposome.

During the monitoring of early-stage separation, we observed a distinct separation between the peak of EOF and the one corresponding to liposome with increased time of applied voltage (**Fig. 14**). In the borate buffer without CTAB, a broad peak with a smaller and narrower component was still observed. As the voltage application time increased, this small peak further separated from the EOF, migrating towards the positively charged electrode. This peak maintained a consistent size throughout the measurements, indicating that no sample aggregation or wall adsorption was occurring. In the borate buffer with CTAB, a reversed voltage was applied, resulting in the liposome peak migrating before the EOF, as expected. Both experimental setups confirmed that liposomes, as negatively charged molecules, migrated towards the positively charged electrode, finally demonstrating the expected electrophoretic behavior.

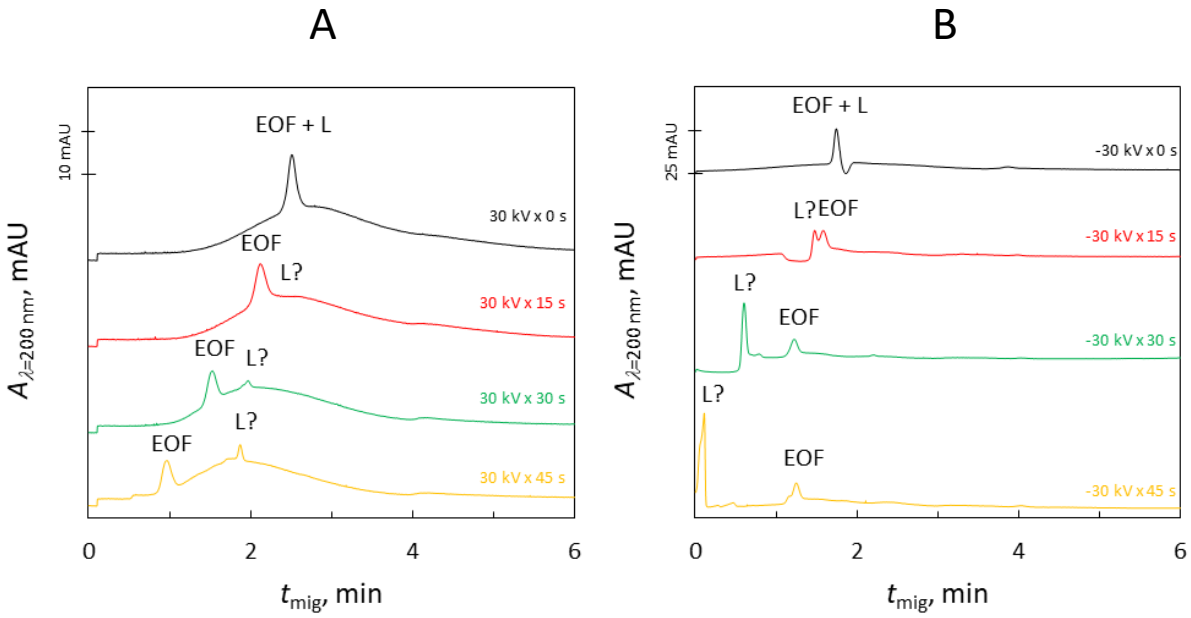



**Fig. 14** – Electropherogram of the DPPC:DPPG liposome with 0.1 mg/ml thiourea with increased time of applied voltage;

BGE: 10 mmol/l borate buffer at pH 9.10 without (A) and with (B) 0.2 mmol/l CTAB. Capillary: 50 µm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec, pressure 10 kPa for 100 sec, applied voltage 30 kV (A) or –30 kV (B) for different time periods and final pressure 10 kPa; (EOF) thiourea, (L?) DPPC:DPPG liposome

We used 5-fluorouracil (5-FU) as a model molecule to study our liposomal formulations; it carries a negative charge in borate buffer at pH 9.10. To enhance the stability of the liposomal sample, we implemented several adjustments. Firstly, we incorporated the lipid DMPE-2000 PEG into the formulation and we switched the hydration buffer from sodium phosphate buffer to 1 mmol/l calcium gluconate with 10% (*w/v*) sucrose [50], which had been reported by Wolkers et al. [80] to increase sample stability. Moreover, due to the shortage of DPPC and DPPG lipids on the market caused by the COVID-19 pandemic, we substituted these lipids with DSPC and DSPG, which should not significantly alter the sample properties. Structurally, DSPC differs from DPPC, and DSPG differs from DPPG by having two additional methylene groups in their alkyl chains. The final molar ratio of DSPC:DSPG:DMPE-2000 PEG lipids was 75:22:3. This sample with encapsulated 5-FU at 0.5 mg/ml concentration was analyzed in the BGE composed of 10 mM borate buffer with 0.2 mM CTAB at pH 9.10. The main peak assigned to 5-FU showed UV spectra characteristic for this API (**Fig. 15**). Additionally, a lower intensity spectrum of 5-FU also appeared in the neutral zone, possibly

due to the interactions between the PEGylated liposomes and CTAB. These interactions could have occurred because CTAB, as a cationic surfactant, may have interacted with a negatively charged liposomes, leading to a modification of their effective charge. As a result, the electrophoretic mobility was affected, and liposomes may have lost their mobility, migrating along with the EOF. Since we did not observe a peak for the encapsulated 5-FU, it is also possible that it has the same mobility as the free 5-FU.

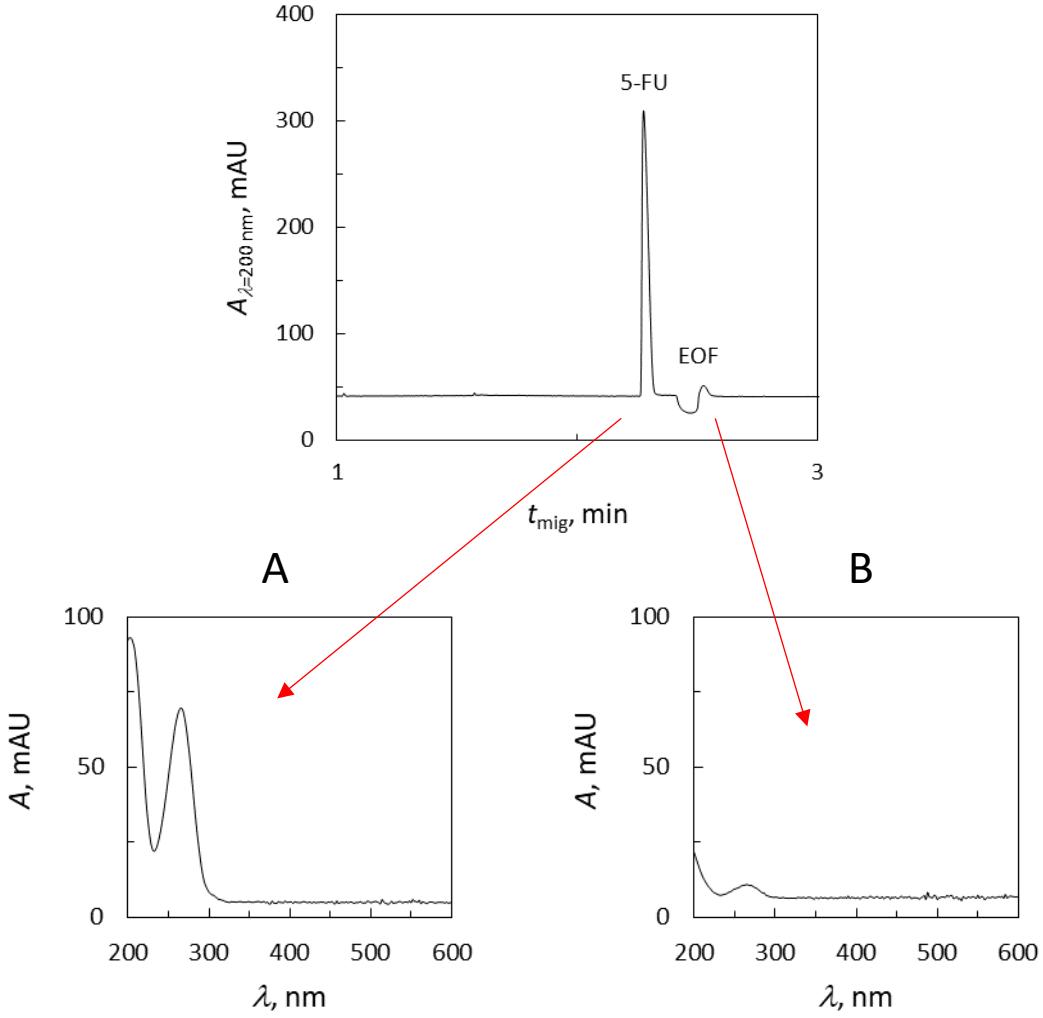



**Fig. 15** – Electropherogram of the DSPC:DSPG:DMPE-2000 PEG liposome with 0.1 mg/ml thiourea and the spectra of 5-FU (A) and EOF (B) peaks;
BGE: 10 mmol/l borate buffer at pH 9.10 with 0.2 mmol/l CTAB. Capillary: 50 µm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec; applied voltage 30 kV; (EOF) thiourea, (5-FU) 5-fluorouracil.

In the process of preparing liposomal formulations, it is crucial to remove encapsulated compounds from the free ones remaining in the aqueous solution after lipid film hydration. This separation is necessary as it ensures accurate characterization of the liposomal formulation, and

it allows us to assess the encapsulation efficiency of given compound. Moreover, unencapsulated compounds could pose a risk to organisms during administration. To achieve this, two methods can be employed. The first method involves removing the supernatant after centrifuging the sample. The second one, which we used, involves fractionation on a solid-phase extraction (SPE) column filled with Sephadex G-25. Large molecules pass through this column while low molecular weight compounds, such as 5-FU, are retained on it. We collected 10 fractions of the eluate, each 0.5 ml, using the same solution as for the preparation of the liposomal sample. The individual fractions were analyzed using the developed CE method (**Fig. 16**). Encapsulated 5-FU eluted in fractions two, three, and four.

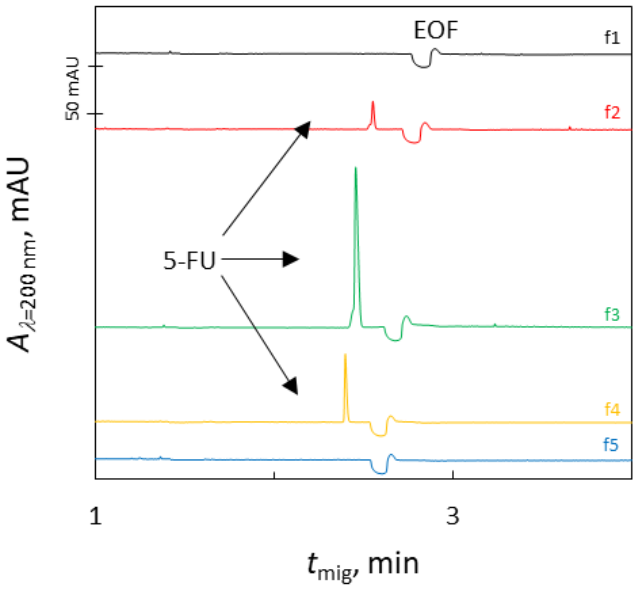



**Fig. 16** – Electropherogram of the individual fractions of the DSPC:DSPG:DMPE-2000 PEG liposome after SPE with 0.1 mg/ml thiourea;
BGE: 10 mmol/l borate buffer at pH 9.10 with 0.2 mmol/l CTAB. Capillary: 50 µm ID, total length 50.0 cm/41.5 cm effective length; sample injection 5 kPa for 5 sec; applied voltage 30 kV; (EOF) thiourea, (5-FU) 5-fluorouracil.

However, we observed an issue with this CE method, as our results indicated that free 5-FU was either not properly separated from encapsulated 5-FU or that free 5-FU may have leaked from the liposomal membrane prior to our CE measurement meaning we did not observe the peak of encapsulated 5-FU at all. Ideally, two distinct peaks of 5-FU should have been present in the electropherogram. One of a free 5-FU that is negatively charged at pH 7.10 and another of an encapsulated 5-FU that should exhibit different mobility. To investigate further, we added the standard of 5-FU to the fraction sample where only encapsulated 5-FU was expected and we observed that the peak of 5-FU increased. Therefore, it was impossible to determine which

form the API belonged to and calculate the EE of this formulation. Due to this ongoing uncertainty and limitations of UV detection, we decided to use a different type of detection, which would allow us to accurately distinguish between the peaks belonging to liposomes and the others.

## 3.2 Laser-induced fluorescence detection

By incorporating a fluorescent tag into the lipid bilayer, we aimed to enhance the detection sensitivity and specificity for the liposomes, thus overcoming the limitations observed with the previous methods. We used fluorescently labeled phosphatidylcholine with 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD). The new sample of liposomes was prepared from DSPC:DSPG:DMPE-2000 PEG:NBD-PC lipids of a total concentration of 5 mg/ml in a molar ratio of 75:22:3:0.5 in 1 mM calcium gluconate solution with 10% (*w/v*) sucrose. The encapsulated molecule remained 5-FU.

Samples without (black line) and with (red line) encapsulated 5-FU were measured in 10 mM borate buffer (**Fig. 17**) without CTAB because we observed interactions between the liposomes and CTAB in the BGE in previous experiments.

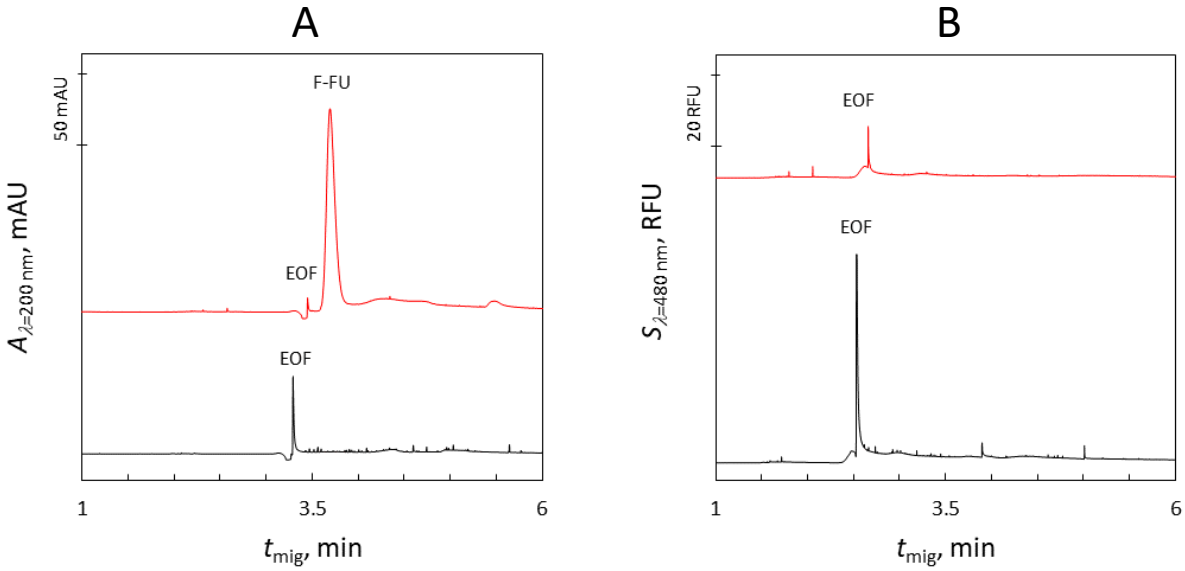



**Fig. 17** – Electropherogram of the DSPC:DSPG:DMPE-2000 PEG:NBD-PC liposome with 0.1 mg/ml thiourea as the EOF marker, without (black) and with (red) encapsulated API; UV detection (A) and LIF detection (B);

BGE: 10 mmol/l borate buffer at pH 9.10; capillary 50 µm ID, total length 50.0 cm/41.5 or 29.0 cm effective length; sample injection 5 kPa for 5 sec; applied voltage 30 kV; (EOF) thiourea, (5-FU) 5-fluorouracil.

Specifically, the neutral zone in the electropherogram contained a lower intensity spectrum of 5-FU, which we attributed to the interactions between PEGylated liposomes and CTAB. Both UV and LIF measurements were performed at the same time, with the LIF detection allowing the identification of fluorescently labeled liposome, while UV detection confirmed the presence of free 5-FU (signal recorded by UV detector but not by LIF detector). However, we did not observe a peak corresponding to liposomes in the LIF signal during conventional CE measurement, possibly due to its adsorption onto the capillary wall.

To shorten the analysis time and thus reduce the risk of liposome adsorption onto the capillary wall, we injected the sample at the short end of the capillary. This required reversing both the pressure and the polarity of the separation voltage. In this setup, as in normal CE mode, the EOF moved towards the detector, while the liposomes migrated in the opposite direction. We measured the sample using the method for monitoring of the early-stage separation (**Fig. 18**) and observed that the peak of liposome kept the same size and was separated further from the neutral zone with increased time of applied voltage.

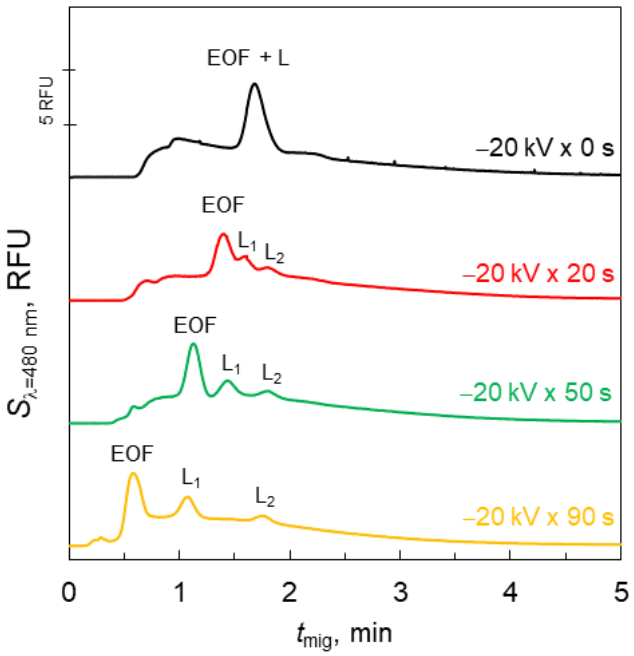



**Fig. 18** – Electropherogram of DSPC:DSPG:DMPE-2000 PEG:NBD-PC liposome with increasing time of applied voltage;

BGE: 10 mmol/l borate buffer at pH 9.10. Capillary: 50 µm ID, total length 50.0 cm/21.0 cm effective length; sample injection -5 kPa for 5 sec, pressure -5 kPa for 30 sec, applied voltage –20 kV for different time periods and final pressure -5 kPa; (EOF) thiourea, (L) liposome.

The peak marked as EOF represents a zone of uncharged sample components that moved at the speed of the EOF, this was verified by recalculating the migration time of thiourea from parallel UV detection signal to theoretical migration time in the LIF detector. However, we observed that using calcium gluconate with sucrose as a hydration buffer might have caused aggregation of the sample resulting in potentially inaccurate results as we observed more than one peak of the liposome. Therefore, we decided to switch back to using 10 mM sodium phosphate buffer for future experiments, as we did not encounter aggregation in this hydration buffer.

We thus prepared a sample of liposome with the same lipid composition, but hydrated in 10 mM sodium phosphate buffer without encapsulated API. We measured this sample by the early-stage separation method in buffers with decreasing pH (**Fig. 19**) to investigate how different pH levels affect the behavior and stability of the liposomes during capillary electrophoresis. With increased time of applied voltage, the EOF peak disappeared because it migrated out of the capillary before the final pressure was applied. A peak corresponding to liposomes was observed in Tris, sodium phosphate and acetate buffers, however, it gradually broadened and then disappeared into the baseline. This proved adsorption to the inner surface of the capillary wall. As the pH of the BGE decreased, the surface charge of the liposome decreased due to the protonation of acidic groups, and their migration aligned with the EOF. This was evident when measuring in phosphoric acid at pH 2.3, where the liposomes remained uncharged and did not migrate out from the EOF peak.

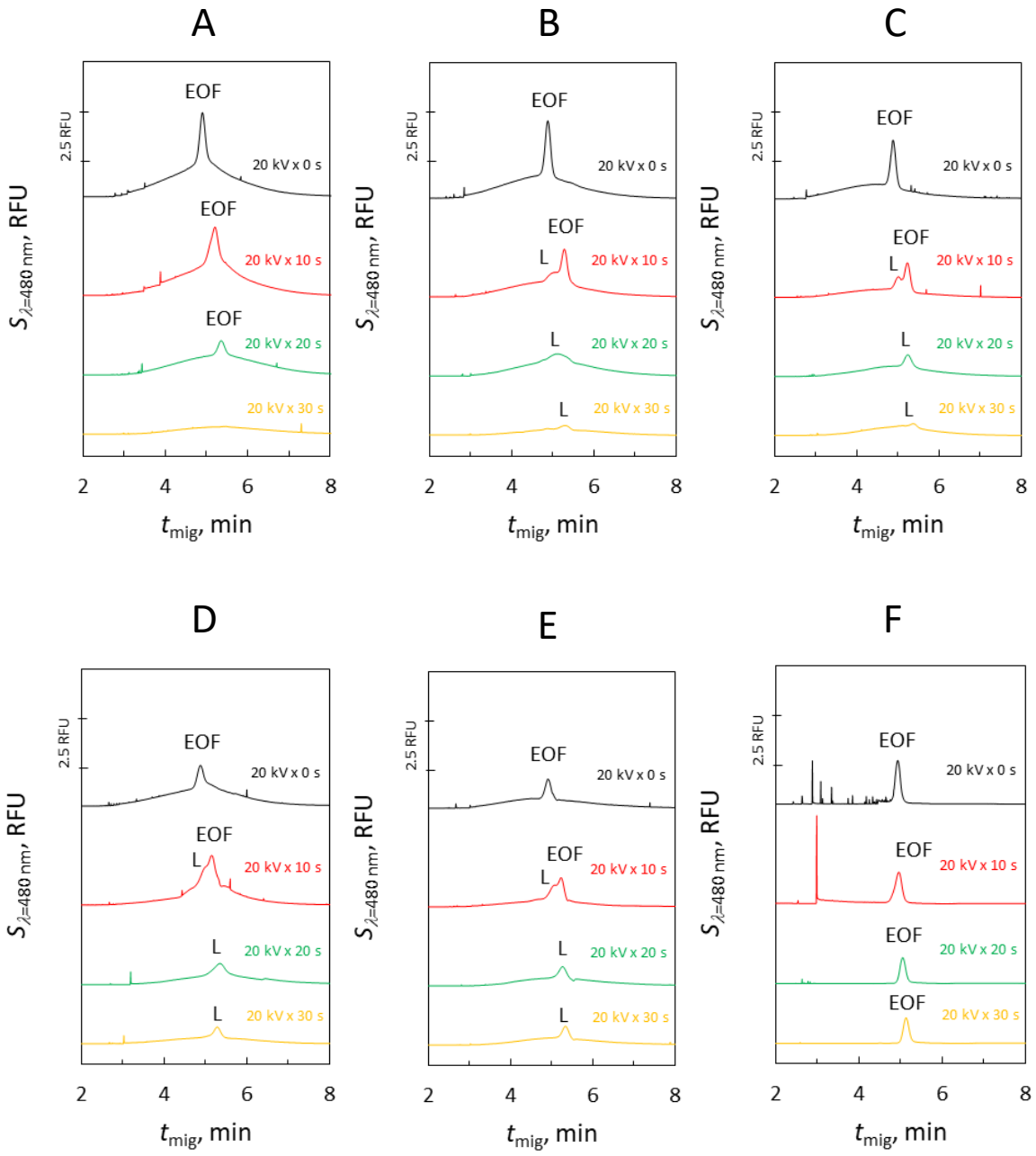



**Fig. 19** – Electropherograms of the fluorescently labeled liposome with increasing time of applied voltage in various BGEs;

BGE: 10 mmol/l borate buffer at pH 9.3 (A), Tris buffer at pH 7.5 (B), 10 mmol/l phosphate buffer at pH 9.0 (C), acetate buffer at pH 5.5 (D), acetate buffer at pH 4.7 (E) and phosphoric acid at pH 2.3 (F). Capillary: total length 50.0 cm/21.0 cm effective length; sample injection 5 kPa for 5 sec, pressure -5 kPa for 30 sec, applied voltage 20 kV for different time periods and final pressure -5 kPa; (EOF) thiourea, (L) liposome.

Based on our measurements, we calculated the mobility $\mu$ and the velocity $v$ of the EOF in all buffers. As shown in **Table 1**, the EOF slows down with decreasing pH of the BGEs, except for the borate buffer. This exception is due to its higher ionic strength $I$, which causes a reduction in the EOF mobility and velocity.

**Table 1** – Experimental data obtained in different buffers

| BGE | pH | $I$, mmol/l | $\mu_{EOF} \cdot 10^8$, $m^2\ s^{-1}\ V^{-1}$ | $v_{EOF} \cdot 10^4$, $m\ s^{-1}$ |
|---|---|---|---|---|
| Borate buffer | 9.3 | 30.0 | 3.77 | 11.6 |
| Tris buffer | 7.5 | 8.0 | 5.38 | 16.5 |
| Phosphate buffer | 7.0 | 20.0 | 4.87 | 15.0 |
| Acetate buffer | 5.5 | 8.5 | 2.76 | 8.51 |
| Acetate buffer | 4.7 | 5.0 | 2.25 | 6.94 |
| Phosphoric acid | 2.3 | 7.6 | 0.75 | 2.32 |

## 3.3 Capillary coating

A more detailed description of all experiments and their results shown in this chapter can be found in the accompanying article in the appendix (**Publication II**); here, we present only selected findings.

As we frequently encountered issues of liposome adsorption onto the inner surface of the capillary, which not only compromised the accuracy of our measurements but also made it difficult to achieve consistent results, we decided to use a commercially coated capillary with polyvinyl alcohol. Using 10 mmol/l sodium phosphate buffer as in our previous experiments (**Fig. 20**), we observed that the liposome peaks shifted to shorter times and maintained their size throughout the entire experiment. This indicated a significant improvement compared to previous experiments with uncoated capillaries, where liposome peaks often broadened or even disappeared.

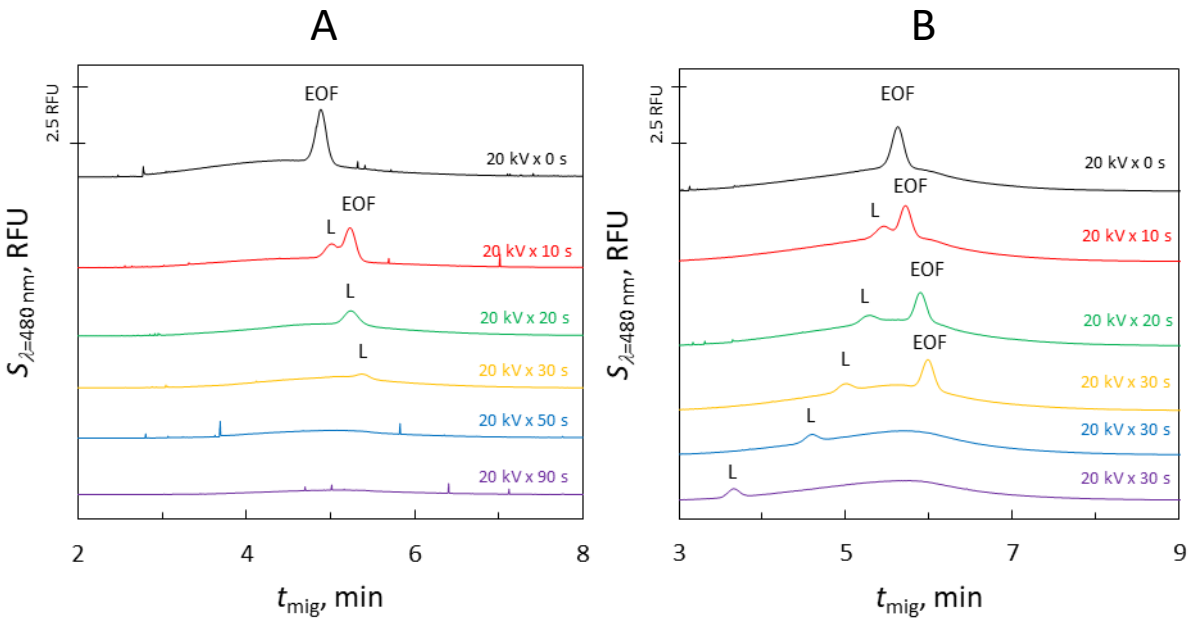



**Fig. 20** – Electropherograms of the fluorescently labeled liposome with increasing time of applied voltage in uncoated (A) and PVA-coated (B) capillary;
BGE: 10 mmol/l sodium phosphate buffer. Capillary: 50 µm ID, total length 50.0 cm/21.0 cm (A) or 64.5/21 cm (B) effective length; sample injection 5 kPa for 5 sec, pressure -5 kPa for 30 sec, applied voltage 20 kV for different time periods and final pressure -5 kPa; (EOF) thiourea, (L) liposome.

Despite the success with the commercially coated PVA capillaries in resolving the adsorption issues, their high cost prompted us to seek more cost-effective alternatives. Our goal was to develop a stable coating method with the suppressed EOF that would enable accurate liposome analysis without relying on expensive commercially coated capillaries.

### 3.3.1 Dynamic coating

For dynamic coating, we explored four different polymers, namely Pluronic F-127 [81], polyvinyl pyrrolidone (PVP) K30 [82], PEG 6000 [83] and polydiallyldimethylammonium chloride (PDADMAC) [84]. These polymers were chosen for their differing charges, polarities, and coating mechanisms, allowing us to optimize the capillary surface for improved liposome analysis. Pluronic F-127 is a non-ionic copolymer surfactant that can effectively reduce interactions with the capillary wall. PVP K30 is a neutral polymer, and it is often used to prevent the adsorption of analytes onto the capillary surface. PEG 6000 is another neutral polymer that provides hydrophilic coating, minimizing the electrostatic interactions between charged analytes and the capillary wall. In contrast, PDADMAC is a cationic polymer that introduces a positive charge onto the capillary surface, reversing the EOF, which is commonly used in the analysis of anions. These polymers were tested by four coating approaches at 1% and 10% (*w/v*) concentrations to identify a cost-effective and stable alternative. Among the methods for dynamic coating, we tried:

· Method I: rinsing the capillary for 10 minutes with a polymer solution before the first measurement;
· Method II: rinsing the capillary for 1 minute with a polymer solution and 1 minute with deionized water between individual measurements;
· Method III: rinsing the capillary for 10 minutes with a polymer solution, applying the voltage of 30 kV for 10 minutes and rinsing it for another 10 minutes with deionized water before the first measurement;
· Method IV: rinsing the capillary for 1 minute with a polymer solution, applying the voltage of 30 kV for 1 minute and rinsing it for 1 minute with deionized water between measurements.

To compare the effectivity and stability of individual coating methods for all four tested polymers, we measured EOF mobility in the coated capillary and expressed it as a percentage of the EOF mobility measured in an uncoated capillary under identical conditions.

The results from coating method I, where the capillary was rinsed for 10 minutes with a 1% solution of given polymer, are shown in **Fig. 21**. The grey column represents the percentage of the mobility in an uncoated capillary (100% by definition), the blue column is the average of the first five measurements in the coated capillary (section 1), the green column is the average of the measurements number eight to twelve (section 2), and purple column is the average of the measurement number sixteen to twenty (section 3).

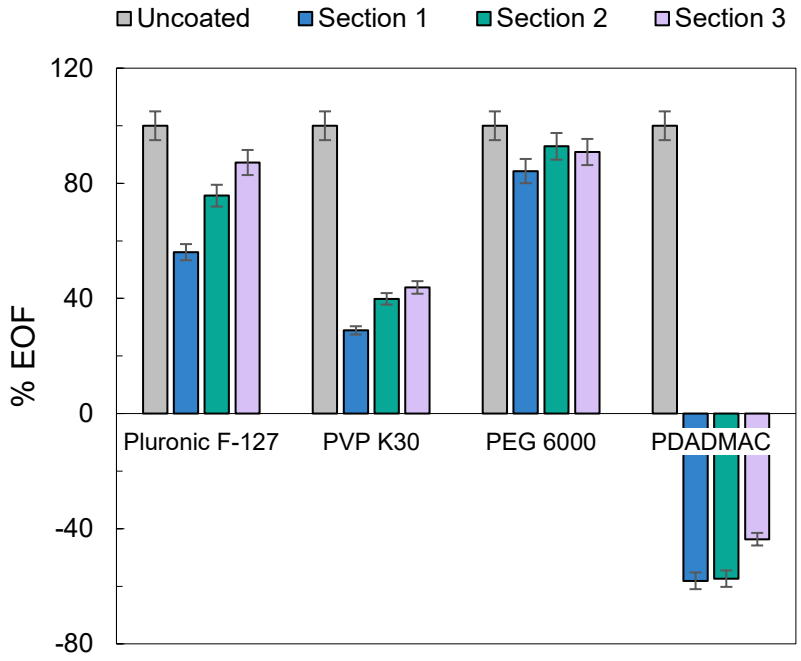



**Fig. 21** – Results from coating method I with a 1% solution of given polymer; BGE: 10 mmol/l sodium phosphate buffer. Capillary: 50 µm ID, total length 33.0 cm/24.5 cm effective length; sample injection 5 kPa for 5 sec, voltage 20 kV (-20 kV for PDADMAC).

In general, method I was effective in the initial EOF suppression, and it demonstrated consistent performance across different polymers, making it a representative choice for comparison. The coating with Pluronic F-127 initially suppressed the EOF, however, it lacked long-term stability as evidenced by the gradual increase in EOF over time. This could be attributed to its tendency to be washed out from the capillary, as its non-covalent attachment is insufficiently strong. In contrast, PVP K30 demonstrated a more stable coating, where the EOF suppression was consistent after a few initial measurements. The weak adsorption of the PEG 6000 onto the capillary surface was evident, leading to insufficient EOF suppression. This was probably caused by its high degree of hydration, which likely prevents it from adhering strongly to the capillary surface. Notably, the cationic nature of PDADMAC imparted a positive charge onto the capillary surface, effectively reversing the EOF. This polymer showed a relatively

stable coating, as its positive charge allowed for strong electrostatic interactions with the negatively charged capillary surface.

Method II showed greater suppression of the EOF, particularly for higher polymer concentrations. However, we observed that the repeated flushing led to either enhanced polymer adsorption or fluctuations in the EOF, depending on the polymer used. For some polymers, Method II demonstrated a more gradual and controlled EOF reduction over time.

Both methods III and IV involved the application of voltage, which, while providing some initial EOF suppression, often led to less stable coatings, particularly when using PEG. Voltage application seemed to disrupt the polymer layer, resulting in reduced EOF suppression effectiveness and stability issues across different polymers.

Among the four mentioned methods used for all four polymers (for more detailed results of each coating see **Publication II**), the most promising one involved flushing the capillary with a 10% solution of PVP K30 before the first measurement. Using this coating, we obtained a good stability of the electroosmotic flow and the mobility decreased about 3.5 times compared to the uncoated capillary. While the 10% PVP K30 solution provided effective EOF suppression, it also resulted in some initial peak deformation due to the excess polymer. Despite this, the method was selected as the most effective for our purposes.

This dynamic coating provided a stable separation environment, reducing liposome adsorption and maintaining peak integrity (**Fig. 22**). Additionally, when the voltage was applied for a longer time, this peak split into two, which might be explained by the polydispersity of the sample, indicating a bimodal size distribution of this liposome batch. Fractionation using the SPE column with Sephadex G-25 confirmed this observation as we detected two individual fractions of liposomes in different eluates. Specifically, one fraction of liposomes representing one size population was observed in eluates two and three, while a second fraction was present in eluates four and five. This highlights the importance of precise and consistent sample preparation to ensure reproducibility and accuracy in measurements. Nevertheless, this simple dynamic coating demonstrated a potential for sufficient analysis of liposomes.

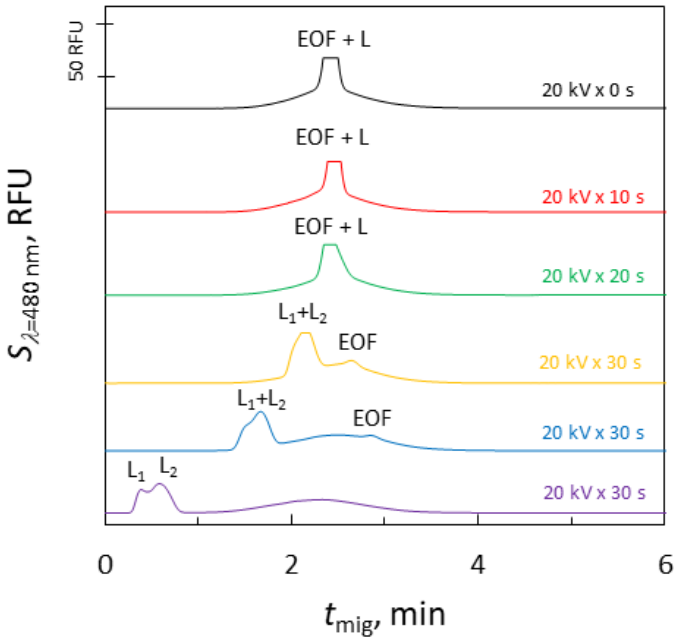



**Fig. 22** – Electropherogram of the fluorescently labeled liposome with increasing time of applied voltage in 10 % PVP-coated capillary by method I;
BGE: 10 mmol/l sodium phosphate buffer. Capillary: 50 µm ID, total length 50.0 cm/21.0 cm effective length; sample injection 5 kPa for 5 sec, pressure -5 kPa for 30 sec, applied voltage 20 kV for different time periods and final pressure -5 kPa; (EOF) thiourea, (L) liposome.

### 3.3.2 Permanent coating

Lastly, we explored the use of a permanent coating with linear polyacrylamide (LPA), which provided even more reliable results. The permanent coating was carried out following the procedure used by Hamidli et al. [85] in electrophoretic separation of proteins. We used LPA coating not only to suppress liposome adsorption to capillary walls but also to effectively eliminate the EOF for accurate measurements of their mobility. The coating suppressed the EOF below 0.40% (calculated by **Equation 3**) of its original value in uncoated capillary (calculated by **Equation 2**) and we observed high stability of the coating. The EOF was initially reduced to 0.26% after the first measurement and after forty measurements, the EOF remained practically completely suppressed at 0.38%.

As we prolonged the voltage application, we again observed a gradual decrease in the migration times of liposomes (**Fig. 23**), a trend consistent with our previous findings. Unlike previous coatings, the peak of liposome was more symmetrical, suggesting that LPA coating was successful in minimizing interactions that typically lead to peak broadening or distortion.

Overall, this permanent coating method provided a stable environment for our electrophoretic analyses, improving the reliability of our measurements.

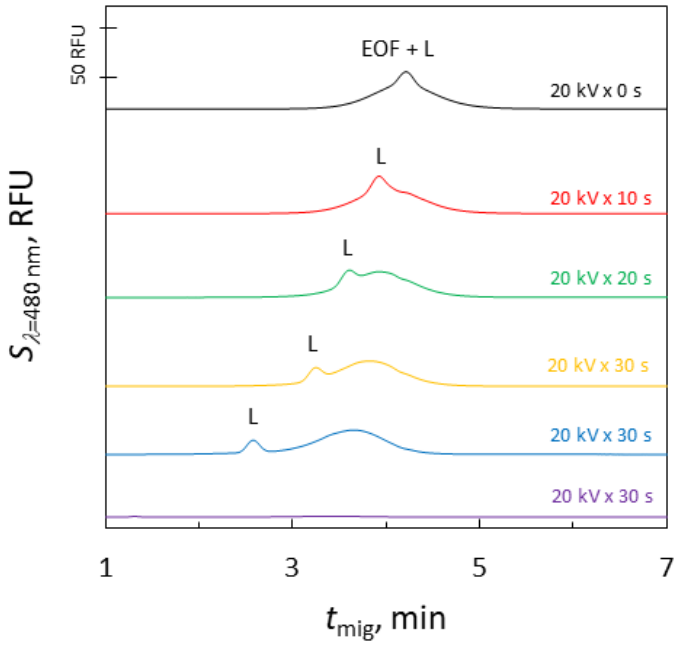



**Fig. 23** – Electropherogram of the fluorescently labeled liposome with increasing time of applied voltage in permanently LPA-coated capillary;
BGE: 10 mmol/l sodium phosphate buffer. Capillary: 50 µm ID, total length 50.0 cm/21.0 cm effective length; sample injection 5 kPa for 5 sec, pressure -5 kPa for 30 sec, applied voltage 20 kV for different time periods and final pressure -5 kPa; (EOF) thiourea, (L) liposome.

### 3.3.3 Conclusion

We evaluated the effectiveness and stability of various coating methods for suppressing and modifying the EOF. The dynamic coating was tested for several polymers, and we used a single-flush coating with a 10% solution of PVP for the liposome analysis. We obtained results comparable with a commercially PVA-coated capillary, but with significantly lower costs. The permanent coating with LPA provided effective and stable EOF suppression, which allowed us to precisely analyze liposome mobility. Though its preparation was time-consuming, it offered a significant improvement of liposome peak shape and minimized interactions with the capillary wall.

## 3.4 API-liposome interactions

### 3.4.1 Preliminary experiments

In the next phase of our work, we have decided to explore a different aspect that is significant for the pharmaceutical application of liposomes: the use of capillary electrophoresis for monitoring API-liposome interactions. Instead of using capillary zone electrophoresis (CZE), two alternative methodologies mentioned previously can be employed. Briefly, in CEC methodology, the capillary is rinsed with a liposome sample, allowing liposomes adsorbed on the capillary wall to serve as a stationary phase. LEKC involves adding liposomes directly to the background electrolyte, where they serve as a pseudostationary phase. Both approaches provide valuable insights into the behavior and interactions of APIs with liposomes. A more detailed description of all experiments and their results can be found in the accompanying manuscript in the appendix (**Publication III**); here, we present only selected findings.

At first, we tested different types of CE vials to reduce liposome sample consumption in the BGE. While smaller vials enable lower liposome consumption, the smaller volume also means that changes in the BGE composition due to electrolysis during the measurement are more pronounced. Typically, vials with a BGE volume of 600 µl are used in our laboratory. For smaller volumes, conical vials with a volume of 200 µl or vials with glass inserts with a volume of 150 µl can be used. The mobility of the electroosmotic flow increased gradually in all vial types, with the lowest relative standard deviations (RSD) observed in classical vials, as expected. This trend was observed across all vial types in BGEs both with and without liposomes, suggesting gradual degradation of the BGEs due to electrolysis. For classical vials, the RSD values for three measurements were 1.53%, while for conical vials it was 6.28% and for glass inserts it was 5.22%. Although the conical vials had a larger BGE volume compared to the glass inserts, their higher RSD value was likely caused by the adsorption of the liposomes onto their polypropylene surface. This adsorption could have led to inconsistent liposome concentrations in the BGE, thereby affecting the reproducibility of the measurements. For these reasons, vials with glass inserts were chosen for LEKC measurements, as they minimize liposome consumption and adsorption, and they provide more consistent results.

After choosing vials with glass inserts as containers for BGE, we proceeded to investigate API-liposome interactions using LEKC. We began with preliminary screening using liposomes that were prepared from DPPC and DPPG in a 75:25 molar ratio, hydrated in 10 mmol/l sodium phosphate buffer. Initially, we tested four lipophilic APIs (canagliflozin,

maraviroc, deferasirox and ambroxol hydrochloride) with varying charges in pH 7.10. The objective was to observe the effects of increasing liposome concentration in the BGE on separation kinetics. We observed that either mobility and/or peak shape changed as the amount of liposome increased. Notably, canagliflozin as a neutral API migrated out of the neutral zone in the presence of the negatively charged liposomes, exhibiting anodic effective mobility, likely due to hydrophobic interactions with liposomes. Positively charged maraviroc maintained its mobility with minor changes in peak shape. Positively charged ambroxol HCl showed significant changes in peak shape even with a small amount of liposomes in the BGE and with higher liposome concentrations, the peak gradually broadened and disappeared in the baseline. Negatively charged deferasirox showed no changes in peak shape or effective mobility in the standard mixture, suggesting no interaction with the liposomal pseudostationary phase. The mobility difference between the negatively charged free API and its complex with liposomes is smaller compared to neutral or positively charged APIs, meaning that any interactions would cause less noticeable changes in mobility. However, when deferasirox was measured alone, the peak shape changed from fronting to tailing, proving certain interaction of deferasirox with liposomes. The effect on the peak shape was absent in the measurements with API mixture likely due to liposome saturation by other APIs (**Fig. 24**). These observations confirmed that LEKC could effectively explore API-liposome interactions.

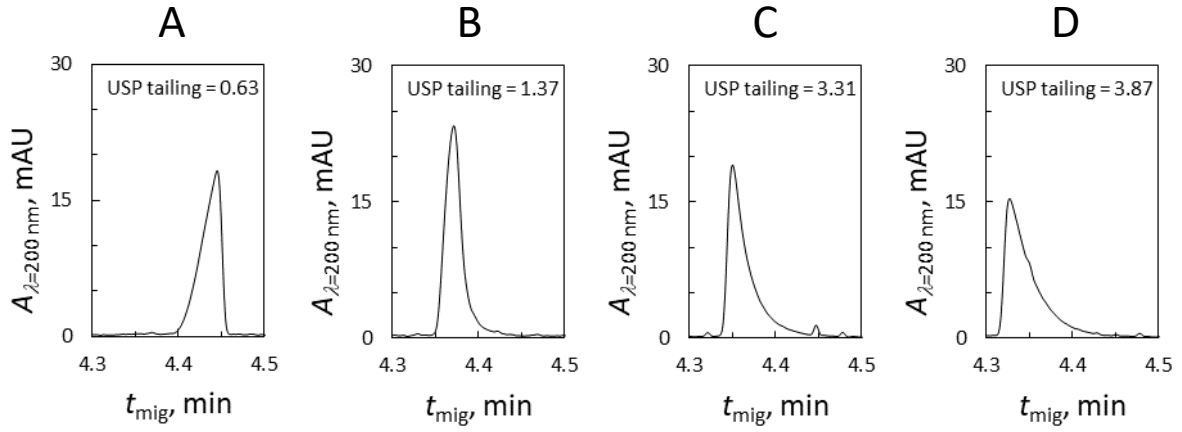
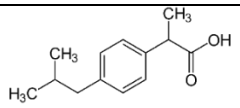
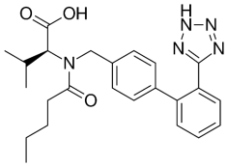
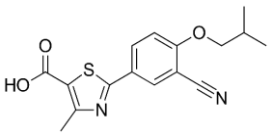
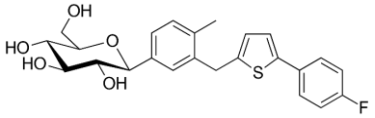
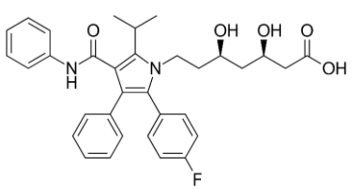


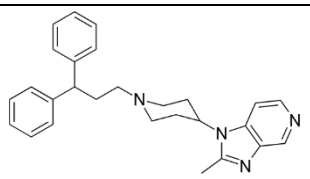
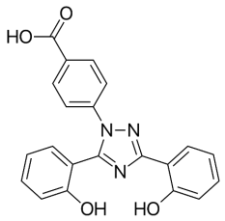
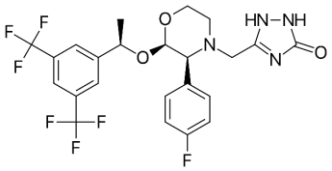
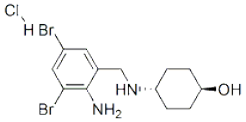

**Fig. 24** – Electropherograms of deferasirox in BGEs with increasing amount of liposomes; BGE: 10 mmol/l sodium phosphate buffer at pH 7.10 (A), 10 mmol/l sodium phosphate buffer at pH 7.10 + 10% of liposomes (B), 10 mmol/l sodium phosphate buffer at pH 7.10 + 20% of liposomes (C), 10 mmol/l sodium phosphate buffer at pH 7.10 + 30% of liposomes (D). Capillary: 50 µm ID, total length 50.0 cm/21.0 cm effective length; sample injection 5 kPa for 5 sec, applied voltage 20 kV

### 3.4.2 The effect of cholesterol content

Next, we prepared four different liposomes with increasing cholesterol content from DPPC, DPPG, and Chol lipids in molar ratios of 75:25:0, 65:25:10, 55:25:20, and 45:25:30, respectively, to evaluate its effect on membrane permeability. Cholesterol is known to modulate membrane fluidity and permeability of the lipid bilayer [13]. In theory, increasing cholesterol content should lead to a more rigid membrane, potentially affecting the interaction of lipophilic APIs with the liposome membrane and thereby altering their electrophoretic mobilities. To test this, we used 9 lipophilic APIs of different properties (**Table 2**). Opposite to our expectations, the increased cholesterol content did not affect peak shapes nor the effective mobilities of individual APIs. The relative standard deviations in BGEs containing 30% liposomes with varying cholesterol compositions were less than 1%. These observations indicated that cholesterol content in our liposomal samples did not impact ongoing interactions.

**Table 2** – Characteristics of used APIs provided by Zentiva, k.s.

| No. | API | *pKa* [86] | log *P* [86] | Charge at pH 7.10 | Chemical structures |
|---|---|---|---|---|---|
| 1 | Ibuprofen | 4.85 | 3.84 | – |  |
| 2 | Valsartan | 4.35 | 5.27 | – |  |
| 3 | Febuxostat | 3.08 | 3.52 | – |  |
| 4 | Canagliflozin | 12.57 | 3.52 | n |  |
| 5 | Atorvastatin Ca | 4.31 | 5.39 | – |  |

| | | | | | |
|---|---|---|---|---|---|
| 6 | Maraviroc | 13.98 | 3.63 | + |  |
| 7 | Deferasirox | 4.51 | 4.74 | – |  |
| 8 | Aprepitant | 6.59 | 5.22 | n |  |
| 9 | Ambroxol HCl | 15.26 | 2.65 | + |  |

### 3.4.3 Liposomes from tissue extracts

To further explore the possibilities of LEKC in terms of developing a more effective method for understanding drug-lipid interactions, we performed a similar experiment using liposomes that were prepared from tissue extracts, specifically from bovine liver or heart (**Fig. 25**). These extracts were chosen because they more closely mimic the *in vivo* conditions, offering a more accurate model for studying drug-lipid interactions in a biological context. Tissue extract-based liposomes were also hydrated in 10 mmol/l sodium phosphate buffer and had a total lipid concentration of 5 mg/ml. Canagliflozin migrated out of the neutral zone more significantly in both liver and heart-based liposomes, with stronger interactions observed in liver-based liposomes. This may be due to the higher amounts of PC and PE in the liver extract, which can increase the hydrophobicity of the liposomal membrane. Additionally, this extract also contains about 5% of Chol, which is known to alter membrane properties, potentially leading to stronger hydrophobic interactions with Canagliflozin. On the other hand, the heart extract composition is less clearly defined, as it contains over 50% of unspecified neutral lipids and about 17% of unknown components. These factors need to be taken into consideration when interpreting the results. Maraviroc showed varied interaction patterns, with liver-based liposomes causing an increase in the tailing factor and rapid reduction in effective mobility,

dropping to 20% of its original value. In contrast, with heart-based liposomes, the tailing factor also increased, but the mobility only decreased to 90% of its original value. These findings suggest a much stronger interaction between maraviroc and the liver extract liposomes. Ambroxol hydrochloride lost its mobility in liver-based liposomes, indicating strong interactions, and required higher concentrations of heart-based liposomes to achieve similar effects. Deferasirox consistently showed no changes in effective mobility across different liposome types but exhibited altered peak shapes when measured alone.

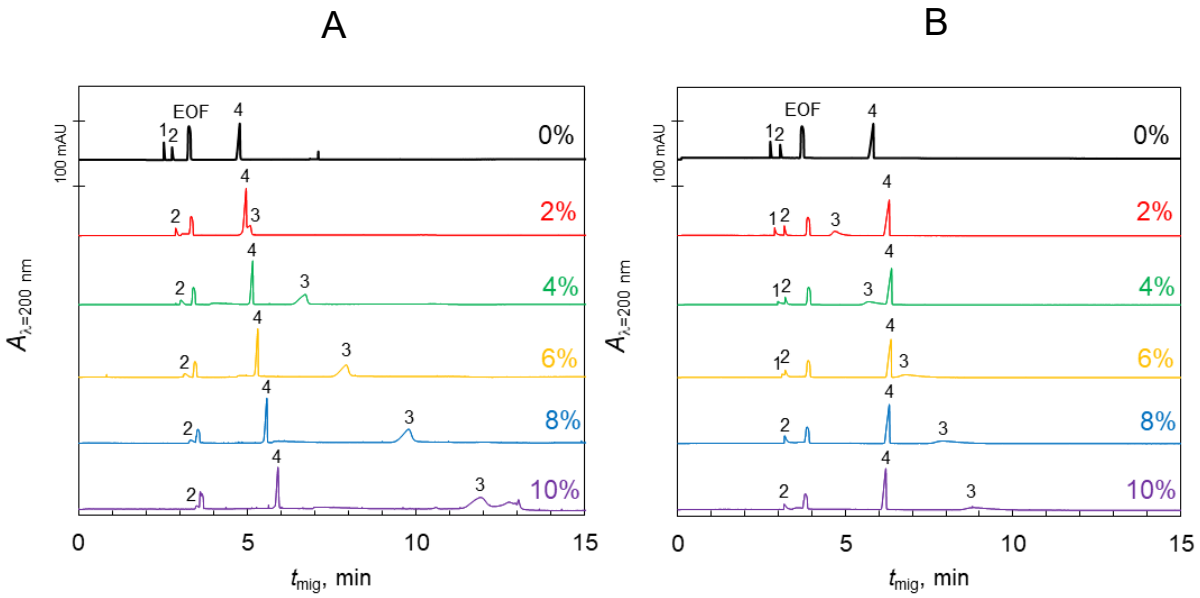



**Fig. 25** – Electropherograms of liposomes from bovine liver (**A**) or bovine heart (**B**) extracts with increasing amount of liposomes in the BGE;
BGE: 10 mmol/l sodium phosphate buffer. Capillary: 50 µm ID, total length 50.0 cm/21.0 cm effective length; sample injection 5 kPa for 5 sec, applied voltage 20 kV; (1) ambroxol hydrochloride, (2) maraviroc, (3) canagliflozin and (4) deferasirox

### 3.4.4 The effect of temperature

We also investigated the impact of temperature on API-liposome interactions for nine lipophilic APIs (**Table 2**). We observed that increasing temperature generally enhanced the effective mobility of most APIs due to decreased viscosity of the BGE and increased fluidity of the liposomal membrane (**Fig. 26**). Canagliflozin exhibited different behavior, in liver-based liposomes, its effective mobility remained unchanged, while in heart-based liposomes, the mobility decreased, suggesting reduced affinity with increasing temperature.

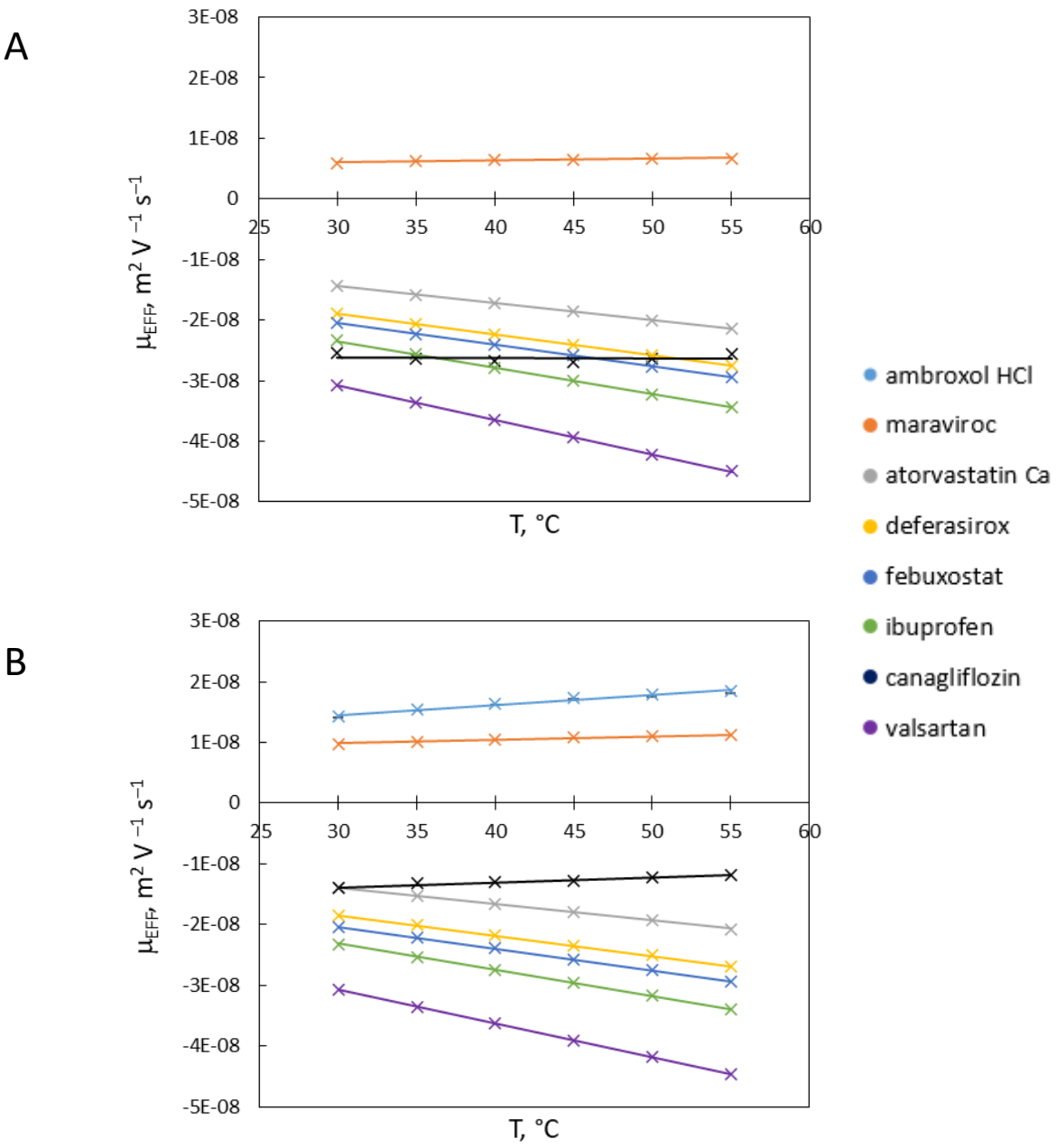



**Fig. 26** – Effective mobilities of individual APIs with increasing temperature during measurements;

BGE: 10 mM sodium phosphate buffer at pH 7.10 with 4 % of liposomes from bovine liver (A) or bovine heart (B) extracts. Error bars are not shown, because the standard deviation values did not exceed $10^{-10}$ $m^2V^{-1}$ $s^{-1}$ for any of the data points.

### 3.4.5 The effect of pH

Finally, the effect of pH on API-liposome interactions was tested using sodium phosphate buffers at 20 mmol/l ionic strength at pH levels ranging from 6.0 to 8.0 in 0.5 increments with the addition of 4% liposomes from tissue extracts. Changes in API behavior were challenging to attribute solely to pH or liposome presence, as both factors significantly impact API ionization and liposome surface charge, complicating the interpretation of results.

### 3.4.6 Conclusion

Our findings highlight the importance of considering lipid composition, temperature, and pH when evaluating API-liposome interactions. These factors significantly influence the separation behavior of APIs and can improve the optimization of liposome-based drug delivery systems. Moreover, positively charged APIs interact more strongly with negatively charged liposomes, likely due to the contribution of electrostatic forces, which result in changes in peak shape and, in some cases, in reduced effective mobility. Negatively charged APIs show weaker interactions, with less pronounced effects on their mobility, likely due to the electrostatic repulsion. Additionally, liposomes prepared from liver extract, which have a higher content of negatively charged components, generally exhibit stronger interactions. This leads to more pronounced changes in the effective mobility and peak shapes of APIs compared to liposomes prepared from heart extracts, though individual differences were observed. We demonstrated the feasibility of using liposomes from tissue extracts as a pseudostationary phase in capillary electrophoresis, offering an approach for developing more effective lipid-based drug delivery systems.

# 4 CONCLUDING REMARKS

In this study, we explored various methodologies and conditions for the analysis and characterization of liposomes using capillary electrophoresis. Our primary objectives were to identify suitable background electrolytes and optimize experimental conditions for liposome analysis and subsequently to minimize liposome adsorption onto the capillary wall.

To address the limitations of the UV detection method, we incorporated fluorescently labeled phosphatidylcholine into our liposomal formulation. This allowed us to distinguish between fluorescently labeled liposomes and free API. By using laser-induced fluorescence detection, we achieved clearer identification of liposome peaks, thereby improving the accuracy of our analyses.

Reoccurring issues with liposome adsorption onto the capillary wall prompted us to explore various coating methods. We investigated both dynamic and permanent coating methods. Among the tested polymers (Pluronic F-127, PVP K30, PEG, and PDADMAC) for dynamic coating, PVP K30 at a 10% concentration coating by the “single flush” method I proved to be the most effective in reducing liposome adsorption and maintaining peak integrity. This method provided a cost-effective alternative to commercially coated capillaries. Permanent coating with linear polyacrylamide significantly suppressed the EOF and minimized interactions leading to peak broadening. Despite being time-consuming, LPA coating offered stable conditions for electrophoretic analysis of liposomes.

We extended our research to examine the interactions between liposomes and various APIs. By utilizing liposomal electrokinetic chromatography, we observed the effects of liposome concentration on API separation kinetics. These interactions can manifest not only as changes of the effective mobility of APIs but also as alterations in the shape of their peaks. Our findings revealed that liposome composition, temperature, and pH significantly influenced API-liposome interactions. We observed several general trends during our measurements, yet there were also individual differences in the behavior of certain APIs. These insights are crucial for optimizing liposome-based drug delivery systems.

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# CONFIRMATION OF PARTICIPATION

1. **Tomnikova, A.**, Orgoníková, A., Křížek, T. (2022) Liposomes: preparation and characterization with a special focus on the application of capillary electrophoresis. *Monatshefte fur Chemie - Chemical Monthly*, *153*, 687-695. 70 %
2. **Šimonová, A.**, Píplová, R., Balouch, M., Štěpánek, F., Křížek, T. (2024) A comprehensive study on capillary surface modifications for electrophoretic separations of liposomes. *Monatshefte fur Chemie - Chemical Monthly*. 70 %
3. **Šimonová, A.**, Balouch, M., Štěpánek, F., Křížek, T. (2024) Investigating drug-liposome interactions using liposomal electrokinetic chromatography. Manuscript submitted to *Analytical and Bioanalytical Chemistry*. 80 %

I confirm that the information given above is true, complete and accurate.

Prague, August 2024 ……...……

doc. RNDr. Tomáš Křížek, Ph.D.

# LIST OF PUBLICATIONS

1. Nesměrák, K., Štícha, M., Belianský, M., **Tomnikova, A.** (2022) Long-term stability of phenobarbital in various pharmaceutical products. *Monatshefte für Chemie - Chemical Monthly*, 153, 735–744.
   DOI: 10.1007/s00706-022-02950-8 $IF_{2022}$=1.8

2. Molnárová, K., Čokrtová, K., **Tomnikova, A.**, Křížek, T., Kozlík, P. (2022) Liquid chromatography and capillary electrophoresis in glycomic and glycoproteomic analysis. *Monatshefte für Chemie - Chemical Monthly, 153*, 659-686.
   DOI: 10.1007/s00706-022-02938-4 $IF_{2022}$=1.8

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# CONFERENCE CONTRIBUTIONS

1. **A. Šimonová**, T. Křížek: Capillary electrophoresis study of active substances and nanoparticles encapsulated in liposomes for the drug development, 3rd Cross-Border Seminar on Electroanalytical Chemistry, 8th-9th April 2021, Online. Oral presentation

2. **A. Šimonová**, T. Křížek, P. Kozlík: Validation of capillary electrophoresis method for determination of monosaccharides found in glycopeptides, 17th International Students Conference "Modern Analytical Chemistry", 16th-17th September 2021, Prague, Czech Republic. Oral presentation

3. **A. Šimonová**, T. Křížek, P. Kozlík: Capillary electrophoresis method for analysis of monosaccharides found in glycopeptides, 1st International Meeting for Young Analytical Chemists, 27th-28th September 2021, Online. Oral presentation

4. **A. Tomnikova**, T. Křížek, M. Balouch: Development of capillary electrophoresis method with laser induced fluorescence detection for liposome analysis, 4th Cross-Border Seminar on Electroanalytical Chemistry, 11th-13th April 2022, Prague, Czech Republic. Oral presentation

5. **A. Tomnikova**, T. Křížek, M. Balouch: Development of capillary electrophoresis method with laser-induced fluorescence detection for liposome analysis, 50th International Symposium and Exposition on High Performance Liquid Phase Separations and Related Techniques, 18th-23rd June 2022, San Diego, USA. Poster presentation

6. **A. Tomnikova**, CE-LIF analysis and characterization of liposomes, 18th International Students Conference "Modern Analytical Chemistry", 15th-16th September 2022, Prague, Czech Republic. Oral presentation

7. **A. Tomnikova**, T. Křížek, V. Ďorďovič: Capillary coatings for liposome analysis by CE, 5th Cross-Border Seminar on Electroanalytical Chemistry, 4th-6th April 2023, Waldmünchen, Germany. *Lecture Award 2023 in recognition of the excellent oral presentation*. Oral presentation

8. **A. Šimonová**, T. Křížek, V. Ďorďovič, M. Balouch: Exploring Coating Agents for Capillary Electrophoresis of Liposomes with Laser-Induced Fluorescence Detection, 51st

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9. A. Dan, H. Vaswani, **A. Šimonová**, A. Grząbka-Zasadzińska, J. Li, K. Sen, S. Paul, Y.-Ch. Tseng, R. Ramachandran: Inline Torque Measurement for End-Point Determination of Heterogeneous Formulations in High-Shear Wet Granulation, Final ORBIS Conference, 5th-6th July 2023, Poznań, Poland. Poster presentation

10. **A. Šimonová**, Exploring Coating Agents for CE with LIF detection for liposome analysis, 21st International Symposium and Summer School on Bioanalysis, 10th-15th July 2023, Târgu Mureș, Romania. Oral presentation

11. **A. Šimonová**, T. Křížek, V. Ďorďovič: Studying the interactions between selected active pharmaceutical ingredients and liposomes by capillary electrophoresis, 19th International Students Conference "Modern Analytical Chemistry", 14th-15th September 2023, Prague, Czech Republic. Oral presentation

12. **A. Šimonová**, T. Křížek, V. Ďorďovič: Capillary electrophoresis analysis of interactions between selected APIs and liposomes prepared from heart and liver extracts, 6th Cross-Border Seminar on Electroanalytical Chemistry, 26th-27th March 2024, Prague, Czech Republic. Oral presentation

13. **A. Šimonová**, T. Křížek: Capillary electrophoresis analysis of interactions between lipophilic active pharmaceutical ingredients and liposomes. 20th International Students Conference "Modern Analytical Chemistry", 19th-20th September 2024, Prague, Czech Republic. Oral presentation

14. **A. Šimonová**, T. Křížek: Analyzing drug-liposome interactions by capillary electrophoresis. 28th International Symposium on Separation Sciences. 22nd-25th September 2024, Messina, Italy. Poster presentation.

## INTERNSHIPS

1. Institut für Pharmazeutische Wissenschaften, Karl-Franzens-Universität, Graz, Austria, program CEEPUS, March 2021, supervisor: ao. Univ. Prof. Dr. Martin Schmid
2. Rutgers, The State University of New Jersey, New Jersey, USA, program ORBIS, November 2022 – February 2023, supervisor: Prof. Rohit Ramachandran, Ph.D.
3. Institute of Molecular Biology – Bulgarian Academy of Sciences, Sofia, Bulgaria, program CEEPUS, May 2023, supervisor: Assoc. Prof. Tamara Pajpanova, Ph.D.

## PEDAGOGIC WORK

1. Participation in the teaching of the "Laboratory Course on Separation Methods", assisting with the method of Determination of cations in urine using capillary electrophoresis. Two-semester course in the years 2020-2024.
2. Consultant for the bachelor thesis of Bc. Nikola Hurychová (defended 2021), Bc. Renata Píplová (defended 2023) and Bc. Petr Stávek (defended 2024); and consultant for the diploma thesis of Mgr. Andrea Orgoníková (defended 2021).
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# APPENDIX

## PUBLICATION I

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REVIEW


# Liposomes: preparation and characterization with a special focus on the application of capillary electrophoresis

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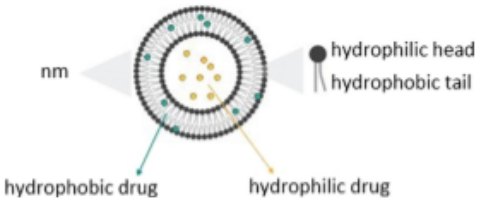
**Abstract**
Liposomes are nowadays a matter of tremendous interest. Due to their amphiphilic character, various substances with different properties can be incorporated into them and they are especially suitable as a model system for controlled transport of bioactive substances and drugs to the final destination in the body; for example, COVID-19 vaccines use liposomes as a carrier of mRNA. Liposomes mimicking composition of various biological membranes can be prepared with a proper choice of the lipids used, which proved to be important tool in the early drug development. This review deals with commonly used methods for the preparation and characterization of liposomes which is essential for their later use. The alternative capillary electrophoresis methods for physico-chemical characterization such as determination of membrane permeability of liposome, its size and charge, and encapsulation efficiency are included. Two different layouts using liposomes to yield more efficient separation of various analytes are also presented, capillary electrochromatography, and liposomal electrokinetic chromatography.

**Graphical abstract**




**Keywords** Analytical method · Capillary zone electrophoresis · Liposomes · Nanoparticles

## Introduction

Liposomes are small spherical vesicles formed by phospholipids and they have been greatly studied since their discovery in 1964, because they can be used to deliver large amounts of substances through the organism to the final destination [1–3]. Liposomes are formed by amphiphilic molecules of lipids that assemble in an aqueous environment to form vesicles that encapsulate the aqueous phase at their center [4].

The main advantage of liposomes, as drug carriers, is their composition, which makes them biocompatible, biodegradable, non-toxic, and able to encapsulate both, hydrophilic and hydrophobic drugs [2]. Hydrophilic drugs are present in the aqueous compartment inside the liposome, while highly lipophilic drugs are trapped in the lipid bilayer. The encapsulation efficiency of lipophilic drugs is always higher compared to the hydrophilic ones, because lipophilic drugs are strongly repelled by the aqueous environment on both sides of the membrane and thus are very tightly incorporated into the hydrophobic part of the membrane [5].

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Liposomes are used as a transport system for many bioactive materials, such as cytostatics, proteins, peptides, enzymes, DNA, or vaccines [6]. By encapsulating the compound into a liposome, the drug is protected from early inactivation and from the enzymatic processes that commonly occur in the body. Encapsulation into liposomes also reduces the exposure of healthy tissues to the drug [2, 7].

Although liposomes can be used to transport a wide variety of substances, their use in practice is less common. The main reasons are their physico-chemical instability due to oxidation and hydrolysis of phospholipids, low solubility in aqueous solutions, short circulation time in the human body caused by rapid detection by the immune system, and leakage of encapsulated drugs [8].

## Composition of liposomes

Naturally occurring phospholipids, such as glycerophospholipids, sphingolipids, polysaccharides, sterols, or synthetic lipids, which show higher stability, are most often used in the preparation of liposomes. The hydrophilic part consists of a phosphoric acid residue to which a small organic molecule can be bound. The molecule may be positively charged, negatively charged, or zwitterionic. Liposomes are most often prepared from zwitterionic phospholipids, for example, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), or from negatively charged phospholipids, such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), and phosphatidylinositol (PI) [4, 9, 10].

Due to the structure of lipids, spontaneous membrane formation occurs in an aqueous medium. The polar parts of phospholipids interact with both external and internal aqueous media, while hydrophobic fatty acid chains interact with each other and their interaction with the aqueous medium is reduced [11, 12].

## Classification of liposomes

### Size and lamellarity

Liposomes are most often classified according to their size and the number of phospholipid bilayers within the liposome (lamellarity). Based on the number of lamellae, liposomes are divided into unilamellar (single phospholipid bilayer), multilamellar (multiple phospholipid bilayers, diameter greater than 0.5 μm), and multivesicular vesicles (smaller vesicles within the main vesicle, diameter greater than 1 μm). Liposomes with a single phospholipid bilayer are then divided into small unilamellar vesicles (SUVs) with a diameter of 20–200 nm, large unilamellar vesicles (LUVs) with a diameter of 100–1000 nm, and giant unilamellar vesicles (GUVs) with a diameter greater than 1000 nm. Multilamellar vesicles (MLVs) usually contain 5–25 phospholipid bilayers and thus are more suitable for encapsulating hydrophobic drugs. On the other hand, LUVs are more suitable for encapsulating hydrophilic drugs, because they have only one phospholipid bilayer and a much larger volume of aqueous solution within the liposome than SUVs [4, 5, 10, 13, 14].

### Composition and use

Based on their composition and use, liposomes can be divided into conventional, cationic, pH-sensitive, long-circulating liposomes, and immunoliposomes [1]. Conventional liposomes, also called the first-generation liposomes, are liposomes containing neutral or negatively charged phospholipids that are used in drug encapsulation studies or as model cell membranes. Their circulation time in the bloodstream is relatively short, because they accumulate in the cells of the immune system [1, 5, 10]. Cationic liposomes composed of positively charged phospholipids are suitable for the transport of negatively charged macromolecules, such as DNA, RNA, or oligonucleotides [1, 5]. pH-sensitive liposomes are destabilized in an acidic environment, which is used for rapid drug release and tumor treatment [15]. Long-circulating liposomes are used to prolong the circulation in the human body, for example by binding a polyethylene glycol (PEG) chain to the phospholipid molecule of liposome. The circulation time can be extended from a few minutes up to several hours, even days in some cases [5, 10]. Finally, immunoliposomes are liposomes, with monoclonal antibodies (or their fragments) bonded to their surface, and thus, they can be detected by cells that have a specific antigen on their surface. Most commonly, long-circulating liposomes are combined with immunoliposomes, where antibodies are bonded to a molecule of PEG [1, 16].

### In vivo behavior

Liposomes can be further divided by parameters affecting their in vivo behavior. These parameters include the flowability of the phospholipid bilayer or the surface charge of the liposome. Lipids have a characteristic phase transition temperature $T_c$. Below this temperature, they are in the gel state, while above this temperature, they are in the fluid state. The flowability of the phospholipid bilayer can be thus affected by using lipids with different $T_c$ values. If lipids with $T_c$ below body temperature are used, the phospholipid bilayer is more fluid, leading to drug leakage from the liposome. On the other hand, liposomes composed of lipids with $T_c$ higher than human body temperature are less fluid, and thus, the drug leakage is suppressed [10, 17].

Another important parameter influencing in vivo behavior of liposomes is the surface charge that may affect the interaction of liposomes with cell membranes. The mechanism of liposome transport into the cells is based on the adsorption to the cell surface and subsequent endocytosis. Negatively charged liposomes are degraded by endocytosis very quickly, while neutral liposomes do not interact with the cells and the drug is thus released extracellularly [17, 18].

## Preparation of liposomes

There are many methods for preparing liposomes and the choice of an appropriate method depends on several factors, such as toxicity and concentration of the encapsulated drug, the type of solution used for dispersion of liposomes, size of the liposomes, preparation costs, and finally the encapsulation efficiency of the method [7, 19].

### Lipid film hydration method

Hydration of a lipid film is one of the most widely used methods for the preparation of liposomes [20]. To facilitate the formation of the phospholipid bilayer, the temperature of the buffer solution and the temperature during the hydration should be higher than the $T_c$ of the lipid with the highest $T_c$. However, this temperature should also be taken into consideration in terms of the possible degradation of active substances encapsulated into liposomes. The MLVs of various sizes are produced by intensive shaking and homogenous unilamellar vesicles of uniform size can be formed by sonication or extrusion [21, 22].

### Ethanol or ether injection method

Another method of liposome preparation is the ethanol injection method, which is based on rapid injection of the lipid suspension into the aqueous phase. The advantages of this method include the simplicity of the operation and the possibility of preparing a large quantity of liposomes [20, 23, 24]. An alternative to the ethanol injection method is the ether injection method, where lipids are dissolved in diethyl ether and injected into the aqueous phase. Thanks to the higher solubility of lipids in ether, liposomes of higher lipid concentrations can be made using this method [2, 20, 23].

### Emulsification method

Finally, liposomes can be prepared by the emulsification method; typically, a technique called reverse-phase evaporation is utilized. This method provides a higher encapsulation efficiency compared to the injection methods [20, 22, 25].

## Stability of liposomes

The main difficulty when dealing with liposomes is their low chemical and physical stability. The chemical stability is affected by either oxidation or hydrolysis of phospholipids [26]. They can be protected from oxidation by the addition of antioxidants, by shortening the time of their exposure to the light, or by performing the preparation in a nitrogen or argon atmosphere [22].

The stability of liposomes can also be affected by their size, composition of the phospholipid bilayer, surface charge, or the method of their preparation. Smaller liposomes can pass more easily through the cell membranes; on the other hand, the encapsulation efficiency is decreased and surface energy increased, resulting in lower liposome stability. By the addition of cholesterol (Chol) into the phospholipid bilayer, the stability of the liposome increases due to lower membrane fluidity and lower risk of aggregation, and at the same time, the permeability of the membrane decreases which leads to lower drug leakage [8].

To ensure longer stability, the liposomes are stored in the form of a dry powder obtained by lyophilization, during which cryoprotective agents, such as sucrose, glucose, or trehalose, are added to the liposome preventing the leakage of the encapsulated drug. This method is used for thermolabile drugs which would be degraded by elevated temperature during the heat-drying method [2, 10].

## Applications of liposomes

Nowadays liposomes are mainly used for the treatment of cancer, fungal infections, for the transport of analgesics, and in the production of viral vaccines. The first liposome-based product approved by the U.S. Food and Drug Administration in 1995 was Doxil®, which contains encapsulated doxorubicin and is used to treat ovarian and breast cancer [27]. As a result of the COVID-19 pandemic, the research and development activities in the field of liposomes are now globally more intensive than ever. Both Pfizer/BioNTech and Moderna COVID-19 vaccines use cationic liposomes as mRNA carriers into cells, because cationic liposomes and negatively charged mRNA form a stable complex [28]. Liposomes in both vaccines are composed of synthetic cationic or ionizable lipids, distearoyl PC, and Chol. The PEG-2000 is bound to one of the lipids in both vaccines and is believed to be responsible for some allergic reactions, as it is known that human body can develop immunity against PEG [28–30].

## Analysis and characterization of liposomes

To ensure the proper function of liposomes as carriers for bioactive substances, it is necessary to characterize their properties, such as size, lamellarity, surface charge, quantitative composition, and encapsulation efficiency [31]. Commonly used methods to analyze and characterize liposomes include $^{31}$P nuclear magnetic resonance (NMR) [32] which is used for studying the membrane fluidity and thermotropic phase transitions, dynamic light scattering (DLS) [33], atomic force microscopy (AFM) [34], fluorescence spectroscopy [35], high-performance liquid chromatography (HPLC) [36], capillary electrophoresis (CE) [4], and others.

### Size and lamellarity

Liposome size is mostly determined by transmission electron microscopy (TEM), DLS, AFM, and size-exclusion chromatography (SEC) [37]. A general disadvantage of the TEM method is that negative staining with osmium oxide or uranyl acetate changes the structure of the liposome [31]. Cryo-TEM represents an alternative that, unlike TEM, does not require the staining and fixation of the samples, which makes it more suitable for size characterization [38].

In the DLS method, the light of a laser beam is scattered depending on the size and shape of the liposomes. By analyzing the fluctuations in light intensity caused by the Brownian motion of liposome particles in solution, the diffusion coefficients can be obtained that are related to the hydrodynamic size of the liposomes [39].

Other methods for liposome size determination are SEC and high-performance SEC. By these methods, liposomes can be separated from free analytes and at the same time divided into groups based on their size [40]. The main disadvantage of SEC is the loss of lipids due to their adsorption in the gel. To overcome this problem, the gel can be saturated with lipids before the analysis using small, sonicated liposomes [40–42].

### Encapsulation efficiency

Encapsulation efficiency is defined as the ratio of the amount of the encapsulated substance in the liposome to the total weighed substance [43]. To determine the encapsulation efficiency, the free drug is separated from the liposome-encapsulated one by dialysis, gel filtration, or centrifugation. However, these methods can cause liposome destruction and release of the encapsulated drug. Among them, dialysis is the most gentle, but also the most time-consuming and instrumentally challenging one [44, 45]. Gel filtration and centrifugation are thus used more often. After the separation of the encapsulated and free drug, the membrane of the liposome is disrupted and the amount of released encapsulated substance is determined by fluorescence spectroscopy, enzymatic, or electrochemical methods [37].

### Quantitative composition

Most of the methods used to determine the quantitative composition, such as the determination of total phosphate content or the amount of Chol in the membrane, are based on the formation of colored products, which can be then determined spectrophotometrically. A method called Bartlett decomposition is used to determine the total phosphate content where the phosphate is transformed to the inorganic form and creates a blue product after reaction with ammonium molybdate [37, 46].

The amount of Chol in liposomes can be determined by enzymatic methods. The hydrolysis of the cholesterol ester produces free Chol, which is then oxidized by cholesterol oxidase to hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenol creating a colored product [37].

## Proliposomes

Attempts to overcome the above-mentioned disadvantages of liposomes (low physical and chemical stability, leakage of encapsulated substance, hydrolysis, oxidation, and aggregation) led in 1986 to the discovery of proliposomes. In contrast to liposomes, proliposomes exhibit high stability. They are dry, free-flowing particles formed by phospholipids, a porous powder, and the drug [47–49]. Upon contact with the aqueous phase or with body fluids, they form a suspension of liposomes. The phospholipids commonly used for proliposome preparation are phosphatidylcholine and phosphatidylglycerol. Proliposomes also contain water-soluble porous carriers such as sorbitol, mannitol, or microcrystalline cellulose [47].

## Liposomes and capillary electrophoresis

Capillary electromigration methods are suitable for the study of liposomes, because they exhibit several advantageous features, such as low sample consumption, fast and efficient separations, and a high degree of automation [4, 50]. Capillary electrophoresis is a method suitable for characterizing the drug–liposome interactions, for determination of encapsulation efficiency, for studying the drug leakage from liposomes, as well as for liposomes characterization (e.g., size, surface charge, and permeability of the phospholipid membrane) [4].

## Determination of membrane permeability

Permeability of liposome membrane is a key parameter that must be determined when studying liposomes to prevent undesired leakage of encapsulated substances during their circulation in the bloodstream [51]. Tsukagoshi et al. [52] analyzed liposomes composed of dipalmitoyl PC with encapsulated dyes, eosin Y and rhodamine B, by CE with chemiluminescence detection. The dependence of the liposome stability and its membrane permeability on the concentration of encapsulated buffer was monitored by the separation, detection, and quantitation of free and encapsulated dye. Liposomes were dispersed in 10 mM carbonate buffer (pH = 9.0), while within them, eosin Y in carbonate or phosphate buffer of different concentrations was encapsulated. The peak area ratio of free eosin Y to encapsulated eosin Y was then calculated from obtained electropherograms. The higher the ratio, the greater was the permeability of the liposome membrane. The peak area ratio, and thus the permeability, was greater when the concentration of encapsulated carbonate buffer was lower than the concentration of carbonate buffer used to disperse the liposomes. The authors hypothesized that water was released from the liposome to equalize the concentration of carbonate buffer to maintain the osmotic balance. This resulted in shrinkage of liposome with subsequent release of the encapsulated dye.

Franzen et al. [53] used CE to study the effectiveness of oxaliplatin encapsulation and the drug leakage from the PEGylated liposome. The liposomes' samples were sonicated by a sonication probe to induce faster leakage of the drug and the electropherograms (Fig. 1) showed an increased peak of free oxaliplatin and decreased peak of encapsulated oxaliplatin with increasing sonication time. The authors assumed that the disappearance of the liposome peak was caused by the complete release of encapsulated oxaliplatin rather than the destruction of the liposome. Using DLS, they found that liposome particle size decreased with increasing sonication time.

## Determination of size and charge

Although CE is not a widely used method for the determination of liposome size, Duffy et al. [54] used CE with laser-induced fluorescence detection (CE-LIF) to study the properties of liposomes, including their size. To eliminate the electroosmotic flow (EOF) and to reduce the adsorption of the liposomes to the capillary wall, the capillary was coated with poly(acryloylaminopropanol). Liposomes prepared from PC, PS, PE, and Chol were dispersed in 2.5 mM sodium tetraborate (pH = 9.3) and the encapsulated substance was 10 μM fluorescein. They were able to calculate the volume of solution captured in liposomes and thus calculate the radius of the liposome from corrected fluorescence

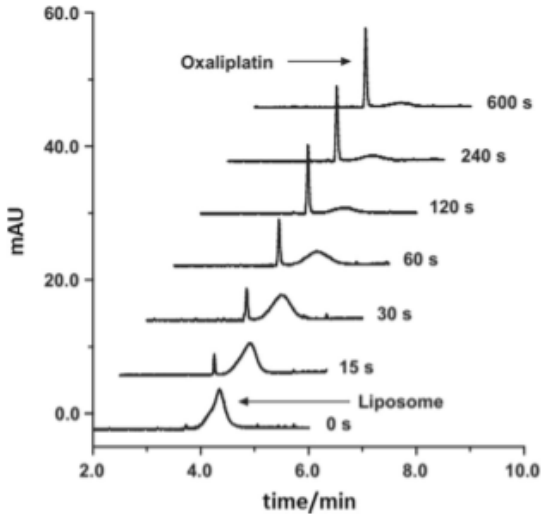


**Fig. 1** Release of encapsulated oxaliplatin from PEGylated liposome with increasing sonication time. Reprinted and modified with permission from Ref. [51]

intensity, fluorescein concentration in the liposomes, and detector sensitivity.

Radko et al. [55] measured mobilities of liposomes composed of PC/PG/Chol in various ratios by extrusion and non-extrusion methods leading to the formation of liposomes with the diameter range from 125 to 488 nm. Based on the measurements of free and encapsulated fluorescein in the capillary coated with 3% non-crosslinked polyacrylamide in the Tris–HCl background electrolyte (BGE) of the different ionic strength, they determined that the electrophoretic mobility is size-dependent (Fig. 2).

For determination of the liposome charge, Wiedmer et al. [56] used CE with UV/Vis detection at 200 nm to measure the electrophoretic mobility of liposomes and DLS to determine the liposome size. The liposomes were composed of palmitoyloleoyl (PO), PC, and PS in various molar ratios and they were dispersed in 50 mM 2-(*N*-cyclohexylamino) ethanesulfonic acid (pH = 9.0). The charge was calculated from electrophoretic mobility, size of the liposome, and the known value of buffer viscosity. The results confirmed the presumption that the higher the content of negatively charged lipids in the liposome, the greater the charge of the liposome.

## Determination of free and encapsulated drugs

Capillary electrophoresis can also be used to study the liposomes as a drug transport system and to determine the amount of free and encapsulated drug [4]. Liposomes are very often used as carriers for toxic drugs, and it is thus

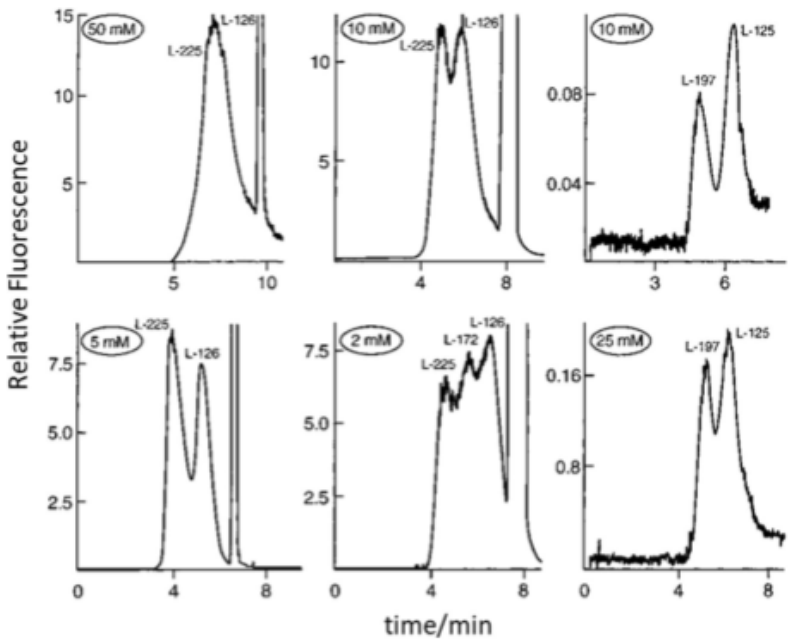


**Fig. 2** Electropherograms showing the size-dependent separation of liposomes varying in their size in 3% linear polyacrylamide gel matrix. Numbers in ovals show ionic strength of Tris–HCl background electrolyte. Reprinted and modified with permission from Ref. [53]

crucial to develop a method for simple and fast determination of encapsulated and free drugs, which could be toxic for the organism. Ansar et al. [57] used CE to separate free and encapsulated doxorubicin in 20 mM phosphate buffer (pH = 6.5) with 10% sucrose as a BGE. Free doxorubicin was determined from the calibration curve by measuring the sample with fluorescein used as an internal standard due to its high stability in phosphate buffer and high absorption coefficient. The total amount of doxorubicin was determined after its release from the liposome using Triton X-100 detergent for membrane disruption.

Another example of the determination of free and encapsulated substances is a study by Chen et al. [58]. They encapsulated oligonucleotides into large neutral or positively charged liposomes, because neutral liposomes do not migrate in the electric field and positively charged liposomes migrate very slowly in the opposite direction than negatively charged oligonucleotides. Free oligonucleotides could thus be separated from the encapsulated ones due to their migration to the anode. To determine total oligonucleotides, liposomes were disrupted by phenol–chloroform extraction, or by Triton X-100 detergent.

Nguyen et al. [59] developed a CE method coupled with MS detection that has a high potential in pharmaceutical quality control and in drug development. They were able to determine the encapsulation efficiency, the stability of liposomes, and the leakage of encapsulated drug from the liposome into the human plasma. The drug release in human plasma was measured before and after incubation of liposome with encapsulated cisplatin (the amount of free and encapsulated cisplatin and total cisplatin were determined in one run), the stability of liposome was measured after incubation at 37 °C, and the triggered release of encapsulated drug into human plasma was measured after sonication.

## Capillary electrochromatography and liposomal electrokinetic chromatography

Apart from being analyzed and characterized by CE, liposomes can also be used to facilitate separations. For this purpose, they can be used in two different layouts, capillary electrochromatography (CEC), or liposomal electrokinetic chromatography (LEKC). CEC combines the features of CE and HPLC. Liposomes are used to coat the capillary wall forming thus a stationary phase. By applying voltage, the EOF is generated and it carries the mobile phase with analytes through the capillary. The charged compounds are separated not only by their different electrophoretic mobilities but also due to their distribution between the mobile and the liposomal stationary phases [60–62]. To coat the capillary wall with liposomes, the capillary can be simply flushed by them, or the avidin–biotin technique can be used. The latter is based on the strong electrostatic interaction between biotin present on the surface of the liposome and the avidin protein, which is bound to the coated capillary wall (usually coated with agarose) [63]. However, the liposomes with biotin are relatively expensive and bound biotin changes the liposome characteristics. Additionally, the avidin–biotin

complex creates a new undesired place where analytes can interact and thus interfere with the drug–lipid interactions in the capillary. Another technique for coating of the capillary wall includes covalent attachment of liposomes after wall activation by 4-nitrophenyl chloroformate [64–66]. The main advantages of CEC over HPLC include higher separation efficiency, low sample consumption, and low analysis costs [61].

Godyń et al. [67] used CEC for studying the blood–brain barrier permeability of different compounds. Prior to the analysis of 25 drug compounds, the capillary was coated with POPC/PS (80:20 mol%) liposomes representing the natural composition of the phospholipid bilayer of the blood–brain barrier. They coated the capillary by flushing it 10 min with 0.5 M HCl, 15 min with water, and then 10 min with liposomes and based on the EOF marker measurements found out that the EOF in the coated capillary was suppressed. They compared the data from CEC to parallel artificial membrane permeability assay as a widely used method. Based on their research and in vivo data of the studied compounds, they concluded that CEC as a relatively fast and inexpensive method could be used as an alternative screening method in early drug development.

In the case of LEKC, the liposomes are added directly to BGE creating a pseudo-stationary phase [68]. This method is based on the same principle as micellar electrokinetic chromatography. The separation of the analytes occurs due to their distribution between the aqueous mobile phase and the liposomal pseudo-stationary phase [69, 70]. Nakamura et al. [71] successfully used the LEKC to separate hydrophobic neutral analytes, namely, biphenyl and naphthalene. Liposomes added to the BGE were composed of dimyristoyl PC and dimyristoyl PG in 10 mM Tris–HCl buffer with 50 mM NaCl (pH = 7.0). Without liposomes in the BGE, both analytes migrated together with EOF and thus were not separated. After the addition of the liposomes to the BGE, the analytes were baseline separated based on their distribution between the two phases.

Wiedmer et al. [72] used LEKC to separate neutral steroid hormones using BGE containing negatively charged liposomes composed of POPC, PS, and Chol of different ratios creating the pseudo-stationary phase. They used bare fused silica capillary despite acknowledging that some quantity of liposomes may be adsorbed to the capillary wall and thus act as a stationary phase, which results in a combination of LEKC and CEC techniques. They also used polyacrylamide coated capillary with suppressed EOF with the same BGEs to avoid liposome adsorption to the wall and faster separation of more hydrophobic steroids. The differences in the interactions between steroids and liposomes with increasing amount of cholesterol were observed.

The main asset of the LEKC is the possibility to simulate and study the interactions of drugs with cell membranes, since liposomes are structurally very similar to them [73]. Lipophilicity plays a significant role when formulating a drug, allowing the drug to pass through the phospholipid bilayer of the membrane. The octanol–water partition coefficient $P_{ow}$ is widely used to quantify the lipophilicity of drugs. However, this parameter is not suitable for the prediction of the drug–membrane interactions. Charged drugs interact with membranes due to both, the hydrophobic and electrostatic interactions. Their $P_{ow}$ value can thus be rather low, but they can still relatively strongly interact with liposomes due to the electrostatic interactions. LEKC proved to be a suitable method for studying the complex interactions between drugs and cell membranes simulated by liposome membranes [74, 75]. Carozzino and Khaledi [73] investigated the influence of the type and concentration of the buffer, its ionic strength, and liposome composition on separation of neutral (phenol) and positively charged (drugs including tetracaine and lidocaine) compounds by LEKC using negatively charged liposomes. They found out that none of the parameters mentioned above affected the partition coefficients of the neutral analytes. On the other hand, the partition coefficients of the positively charged analytes decreased with increasing ionic strength, because the electrostatic interactions between analytes and the liposomes were screened by their interactions with the higher number of counter-ions in the solution. At the same time, the $P_{ow}$ partition coefficient increased with the increasing content of negatively charged lipids present in liposomes, because the electrostatic interaction between positively charged liposomes and negatively charged liposomes increased. All liposomes used to study the effect of liposome composition were prepared with the same concentration of Chol (30% of total lipid concentration) and different combinations of other phospholipids such as dipalmitoyl PC, dipalmitoyl PG, dipalmitoyl PS, dipalmitoyl PE, phosphatidylinositol, and sphingomyelin.

Burns and Khaledi [68] developed a fast LEKC method for determination of liposome–water partition coefficient $K_{lw}$ which better corresponds with cell membranes, as the composition and structure of liposomes are closer to cell membranes than to octanol. The method utilizes the fact that LEKC retention factors calculated from migration times are directly proportional to liposome–water partition coefficient. Studying drug–membrane interactions by LEKC takes only few minutes in opposite to conventional methods, which can take up to days. To validate the method, the authors calculated $K_{lw}$ values from two quantitative structure–partition relationship models and showed great agreement between the experimentally obtained and predicted values. One of the two models was based on the relationship between $K_{lw}$ and $P_{ow}$ and the other was the linear solvation energy relationship (LSER). The strong correlation between the $K_{lw}$ values

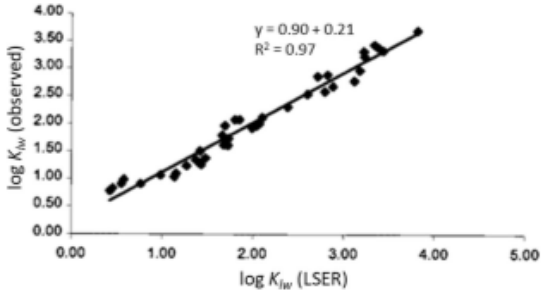


**Fig. 3** Correlation between the $K_{lw}$ values of uncharged aromatic compounds obtained from LEKC and predicted by LSER. Reprinted and modified with permission from Ref. [66]

for uncharged aromatic compounds obtained from LEKC measurements and from LSER prediction is shown in Fig. 3.

Ruokonen et al. [76] studied the effect of temperature (at 25, 37, and 42 °C) on distribution constants $K_{lw}$ of local anesthetics by LEKC using liposomes of three different compositions as pseudo-stationary phase. One type of the liposomes was formed by lipids from human red blood cells for better correspondence with the natural composition of cell membranes. No correlation between temperature and distribution constant of anesthetics was found for any of the tested liposomes; however, the $K_{lw}$ values slightly increased with elevated temperature in most cases. On the other hand, they determined that the $K_{lw}$ values are dependent on the amount of Chol and charged lipids, and surprisingly, the data obtained when using LEKC with red blood cells liposomes showed up to three times lower $K_{lw}$ constants than when using liposomes composed of POPC/POPG or POPC/POPG/Chol, indicating that selection of liposomes for LEKC plays a significant role when conclusions for the in vivo interactions of drugs with cell membranes are to be drawn.

## Conclusion

The properties of liposomes, such as their composition, size, and preparation technology, determine their overall stability, their behavior in the body, as well as their utility as drug carriers. These parameters are crucial in the early drug development and quality control of new products. Capillary electrophoresis methods proved to be promising alternative to the commonly used ones. The main advantages of CE include the possibility of studying the liposome–drug interactions directly inside the separation capillary, which combined with the high degree of automation, several detection techniques available, and short analysis times, make CE a versatile tool for comprehensive analysis and characterization of liposomes. The low sample and buffer consumption, along with the scarce use of organic solvents, results in low operational costs and environmental friendliness of CE methods. Based on the above-mentioned, further development and increasing number of CE applications can be expected, especially in the area of LEKC modeling of drug–cell membrane interactions and LEKC investigation of controlled drug release.

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ORIGINAL PAPER

# A comprehensive study on capillary surface modifications for electrophoretic separations of liposomes

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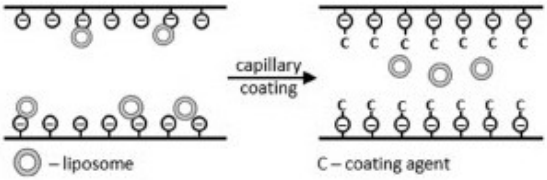
## Abstract

Electroosmotic flow significantly impacts the resolution of separations in capillary electrophoresis and its modification is often necessary. Coating of the inner capillary surface either dynamically or permanently offers a way to alter the electroosmotic flow, potentially reducing the adsorption of analytes, in our case liposomes, to the capillary wall. At first, we measured fluorescently labeled liposomes in an uncoated capillary by capillary electrophoresis with laser-induced fluorescence detection. We used a special procedure of the electrophoretic experiment allowing us to observe the development of peak shape at the early stages of migration. We proved that the liposomes were adsorbed to the capillary wall, which led to a very quick and severe dispersion of their peak during their electromigration. For this reason, we used a commercially coated capillary with polyvinyl alcohol, where at the same separation conditions, we observed the peak of the liposome with a stable shape during the migration. However, this capillary is costly, thus four simple dynamic coating methods were tested for four polymers, namely Pluronic F-127, polyvinyl pyrrolidone K30, polyethylene glycol, and polydiallyldimethylammonium chloride. Among them, we chose a method where we flushed the capillary with a 10% solution of polyvinyl pyrrolidone K30 before the first measurement. In addition, in-house made permanent coating with linear polyacrylamide was investigated, which has led to effective suppression of the electroosmotic flow and stable liposome peak, not dispersed during its migration. Liposome separation using this coating fully confirmed that the studied liposomes are negatively charged and migrate in the anodic direction.

## Graphical abstract




**Keywords** Capillary coating · Capillary electrophoresis · Laser-induced fluorescence · Liposomes

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## Introduction

Capillary electrophoresis offers several advantages for the study, characterization, and analysis of liposomes, including low sample consumption, fast and efficient separations, and a high degree of automation [1, 2]. Capillary electrophoresis has been used for determination of liposome membrane permeability [3, 4], size [5], and charge [6]. Free and encapsulated drugs, i.e., encapsulation efficiency, have been successfully determined using this analytical technique [7–9]. Liposomes

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often exhibit a strong tendency to adsorb to inner capillary walls when bare fused-silica capillaries are used. This phenomenon is utilized in the methods of liposomal electrokinetic chromatography and liposomal capillary electrochromatography where liposomes adsorbed to the capillary wall and/or present in the background electrolyte (BGE) solution serve as a pseudostationary phase, facilitating challenging separations [10, 11]. Liposomes adsorbed on the capillary wall or added to BGE have been used as model systems to study blood–brain barrier permeability for different compounds [12] or to determine the liposome-water partition coefficient as an estimate of partition of drugs between aqueous environment and cell membrane [13]. Although in such cases adsorption of liposomes can be desirable, it usually deteriorates or hinders the measurement and often needs to be eliminated.

To prevent the adsorption of liposomes, and analytes in general, onto silanol groups on the surface of the capillary leading to undesirable broadening of analyte zones, the inner surface can be modified. Suitable capillary coating eliminates unwanted interactions between the analyte and silanol groups and its uniformity and reproducibility play a crucial role. There are three main types of capillary coatings: dynamic, permanent, and hybrid [14–16]. Additionally, coating the capillary surface changes the mobility of electroosmotic flow (EOF), which is also associated with analysis time and peak broadening. Minor changes in EOF can be achieved by adjusting the ionic strength or viscosity of the BGE, major ones by altering the pH of the BGE where a significant decrease of the pH can entirely suppress the EOF. However, these changes may compromise the separation, thus EOF is often modified by capillary coating [15, 17].

The stability of the coating can be determined by repeated measurements of the EOF and by monitoring its changes over time. Several methods for EOF measurements have been published and the basic one involves the addition of a neutral marker that does not exhibit its own electrophoretic mobility [18]. More sophisticated methods also utilize a neutral marker, for example, at low EOF mobility, the method by Williams and Vigh [19] is suitable for determining the EOF. In this method, two zones of a neutral marker are one by one injected by pressure into the capillary, separated by a long zone of the BGE, the voltage is applied on the capillary, causing both zones of the marker to move through the capillary at the mobility of the EOF. Subsequently, a third zone of the neutral marker is injected and all three zones are mobilized by the pressure to pass through the detector. Migration times of the individual zones are used to determine the EOF mobility value as follows:

$$\mu_{\mathrm{EOF}} = \frac{(t_3 - 2t_2 + t_1) l_d l_t}{t_U U t_3}$$

where $t_1$, $t_2$, and $t_3$ are migration times of individual thiourea zones [s], $l_d$ is length of the capillary to the detector [m], $l_t$ is total length of the capillary [m], $t_U$ is time of applied voltage [s], and $U$ is applied voltage [V].

## Dynamic coating

Dynamic coating is a simple and fast method for modifying the EOF. It involves flushing the capillary with a solution of coating agent resulting in capillary coating and it primarily relies on the secondary adsorption interactions of the coating agent with the inner surface of the capillary. To prevent gradual washing out, a small amount of the coating agent can be added into the BGE. However, this approach is not suitable for liposomes as polymers could potentially disrupt or interfere with them. Most common agents for dynamic coatings are polymers, which can be either charged or neutral. Neutral polymers, including polyvinyl alcohol, polyvinylpyrrolidone, polyethylene glycol, acrylamide copolymers, and polysaccharide derivatives, can effectively suppress the EOF. Positively charged polymers are used to reverse the direction of the EOF, while negatively charged polymers can speed up the EOF [16, 20].

Dynamic coating can also be achieved by adding surfactants into the BGE. Commonly used ones are cetyltrimethylammonium bromide or bis(1-dodecyldimethylammonium) dibromide and they reverse the direction of the EOF by forming a double layer with a positive charge on the capillary surface and thus reduce the adsorption of analytes on the capillary wall [21].

## Permanent coating

Permanent capillary coating represents a highly effective method for modifying the EOF and thus suppressing undesired interactions between analytes and capillary wall. This type of coating provides higher stability than dynamic coating, due to the strong covalent interaction between capillary surface and coating agent. On the other hand, it is a more time-consuming process involving three steps, including capillary surface modification, silanization, and covalent attachment of the polymer [16, 22].

The first step leads to activation of the surface of the capillary wall that is necessary for subsequent reactions, it is optimal to initially activate the inner surface with hydrochloric acid followed by sodium hydroxide solution. To enhance the silanization efficiency, it is recommended to completely remove solutions from the capillary by drying with an inert gas. During silanization, a reaction between the silanol groups on the capillary surface and the silanizing agent occurs. During polymerization, radicals are first formed from monomers using an initiator, then they bond together forming a polymer chain. In classical polymerization, the reaction occurs throughout the entire capillary space, and unreacted monomers may remain trapped within the polymer

layer causing uneven coating thickness. To overcome this problem, some polymerization methods use linear polymers growing only from the capillary surface [16].

One of those methods was published by Hjertén [23] and it is based on coating the capillary with linear polyacrylamide (LPA). The inner surface is first silanized with 3-(trimethoxysilyl)propyl methacrylate, whose methoxy groups bind to the silanol ones on the surface. Through a radical polymerization mechanism, acrylate monomers are gradually attached to the acrylate residue of 3-(trimethoxysilyl)-propyl methacrylate, forming a linear polymer. Ammonium or potassium persulfate serves as an initiation agent for the polymerization, and *N,N,N′,N′*-tetramethylethylenediamine is added as a catalyst.

The aim of this work was to identify a suitable coating of the inner capillary surface for the electrophoretic separation of liposomes. Several methods of dynamic and permanent capillary coatings utilizing different polymers as coating agents were tested for capillary surface modification. Specifically, we evaluated the performance of these coatings through repeated measurements of the EOF using thiourea as a neutral marker. One dynamic and one permanent coating were tested for the separation of liposomes, with a focus on their adsorption behavior. Investigation of coating properties concerning the separations of liposomes in capillary electrophoresis can bring important insights for future applications, such as the characterization of liposomes in liposomal pharmaceutical formulations, or liposomes used as synthetic models for the study of extracellular lipidic vesicles.

## Results and discussion

For all types of tested coatings, the effectiveness of the EOF suppression along with its stability were monitored. Utilizing thiourea at 0.1 mg $cm^{-3}$ concentration as a neutral marker, the corresponding values of the EOF mobility were determined in a 10 mmol $dm^{-3}$ sodium phosphate buffer at pH = 7.10, serving as background electrolyte (BGE), for twenty repeated measurements to observe the coating stability. For evaluation, three parameters were selected, specifically median value, standard deviation, and relative standard deviation of EOF mobility for three sections, specifically for measurements 1 to 5 (Sect. 1); 8 to 12 (Sect. 2), and 16 to 20 (Sect. 3). The obtained EOF values were expressed as percentages of the EOF mobility value in the uncoated capillary, whose median was determined to be $(5.25 \pm 0.09) \times 10^{-8}$ $m^2$ $V^{-1}$ $s^{-1}$ (n = 5, RSD = 1.81%).

For dynamic coating, four coating methods were tested for four coating agents at 1% and/or 10% (*w*/*v*) concentration:

Method I. The capillary was once flushed with coating agent solution for 10 min.

Method II. The capillary was flushed with coating agent solution for 60 s followed by flushing it with deionized water for 60 s before each measurement.

Method III. The capillary was once flushed with coating agent solution for 10 min, then a voltage of 30 kV for 10 min was applied with both ends of the capillary immersed in vials containing the coating agent solution, and finally it was flushed with deionized water for 5 min.

Method IV. The capillary was flushed with coating agent solution for 60 s, then a voltage of 30 kV for 60 s was applied with both ends of the capillary immersed in vials containing the coating agent, and finally it was flushed with deionized water for 60 s before each measurement.

### Dynamic coating with Pluronic F-127

The coating obtained by method I (Fig. 1a) for Pluronic F-127 did not exhibit long-term stability for either 1% or 10% polymer solution. Initially, the 10% solution resulted in more effective EOF suppression, however, the EOF almost returned to its original value due to the washing out of the polymer from the capillary after twenty measurements.

Regarding method II (Fig. 1b), an improvement in capillary coating stability was observed, particularly for the 10% solution, where the EOF was suppressed to 21.0%. For the 1% solution, a sudden change in the EOF mobility values occurred after replacing the BGE with a fresh one. The EOF was suppressed less compared to method I, probably due to the better initial adsorption of the polymer to the activated silanol groups and longer duration of the flushing in method I.

Method III (Fig. 1c) resulted in zero suppression of the EOF for the 1% solution. Prolonged voltage application may have disrupted the coating; hence, this method was not tested for the 10% solution.

In the case of method IV (Fig. 1d), EOF suppression was weaker than for method II (identical conditions without application of voltage). This suggests that even a brief voltage application hinders Pluronic F-127 adsorption on the capillary, leading to reduced EOF suppression and ultimately worsening the stability of the coating.

### Dynamic coating with polyvinyl pyrrolidone K30

In the case of coating by method I (Fig. 2a) using both 1% and 10% solution of polyvinyl pyrrolidone (PVP), a relatively stable coating was achieved. It was more significant for the 10% solution but neither showed as intensive washing out effect as was observed with Pluoronic F-127. However, using the 10% solution led to the deformation of the thiourea peak in the initial measurements due to an excess amount of

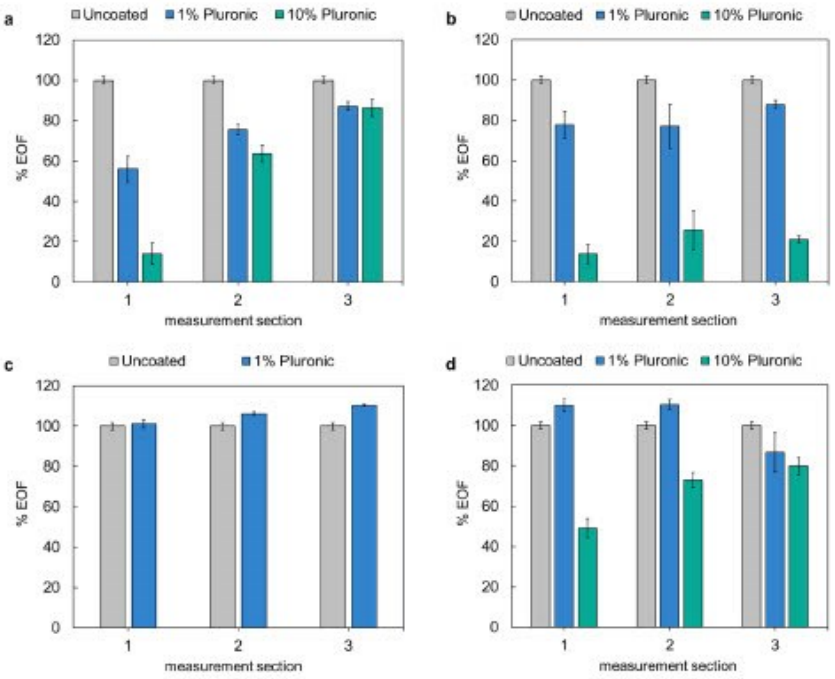


**Fig. 1** Comparison of the EOF suppression and its stability achieved by individual coating methods. Method I (**a**), method II (**b**), method III (**c**), and method IV (**d**) either with 1% (*w/v*) (blue) or 10% (*w/v*) (green) solution of Pluronic F-127. EOF values are presented as medians for each section in comparison to the EOF in an uncoated capillary (grey)

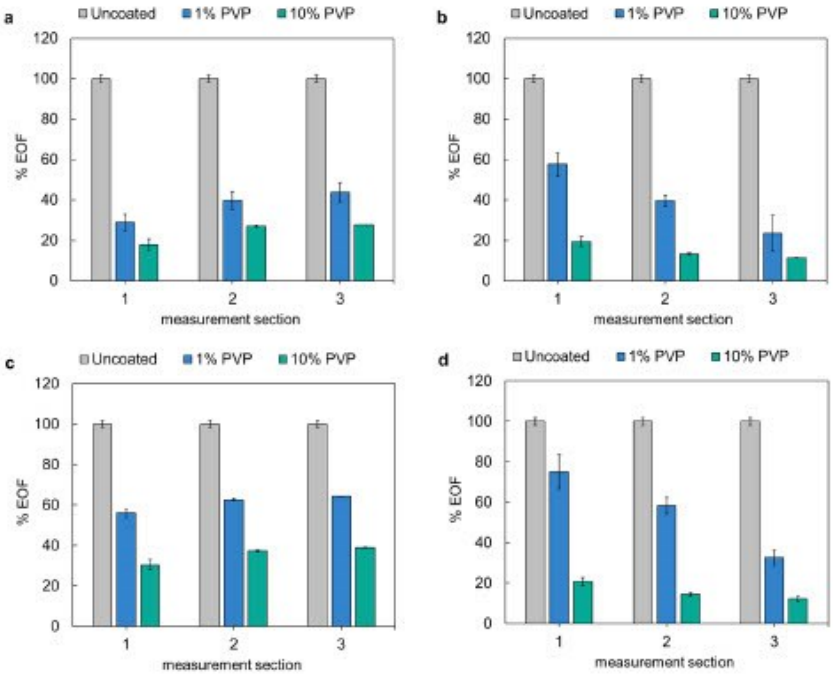


**Fig. 2** Comparison of the EOF suppression and its stability by individual coating methods. Method I (**a**), method II (**b**), method III (**c**), and method IV (**d**) either with 1% (*w/v*) (blue) or 10% (*w/v*) (green) solution of polyvinyl pyrrolidone K30. EOF values are presented as medians for each section in comparison to the EOF in an uncoated capillary (grey)

polymer. Nonetheless, it was selected as one of the potential methods for liposome separation.

Gradually decreasing EOF trend was observed when employing method II (Fig. 2b) for both polymer concentrations. This suggests that each flushing caused increasing adsorption of the agent on the capillary wall. Peak deformation also occurred when using the 10% solution, and due to the consistent decrease of the EOF over time, the coating is unstable, making this method unsuitable for liposome analysis.

The application of voltage had a similar effect for both methods; a one-time application by method III (Fig. 2c) and repeated application before each measurement by method IV (Fig. 2d) resulted in a smaller reduction of the EOF compared to using only capillary flushing. When using the 1% solution and applying the voltage before the first measurement, the stability of the coating increased. However, this trend was not observed for the 10% solution. Repeated voltage application after each capillary flushing had no effect on gradual EOF reduction and the suppression was also less pronounced.

## Dynamic coating with polyethylene glycol

There was no significant suppression of the EOF when utilizing method I (Fig. 3a) for 1% polyethylene glycol (PEG). PEG was gradually washed out and the coating did not exhibit good stability.

Similarly, no significant EOF suppression was achieved when using method II (Fig. 3b). The small difference between the percentage of the EOF in coated and uncoated capillary suggests that PEG does not sufficiently adsorb onto the capillary surface.

The same issue with the insufficient EOF suppression persisted when the voltage was applied after the capillary flushing, whether done one-time by method III (Fig. 3c) or repeatedly by method IV (Fig. 3d). Experiments with Pluronic F-127 and PVP K30 suggested that voltage application may disrupt or damage the formed polymer layer. However, in case of PEG minor coating effect was observed for method IV as the original EOF was suppressed to approximately 80%.

Since the EOF measurements across all four methods indicated insufficient adsorption of the 1% PEG onto the capillary, experiments with a 10% solution were not conducted, as similar behavior regarding polymer washing out was expected. The very weak adsorption of PEG compared to the other neutral coatings tested can be due to the high degree of hydration of this polymer that creates a barrier hindering its adsorption to the capillary.

## Dynamic coating with polydiallyldimethylammonium chloride

Polydiallyldimethylammonium chloride (PDMAC) is the only polymer tested exhibiting a positive charge, resulting in a reversal of the EOF direction upon capillary coating.

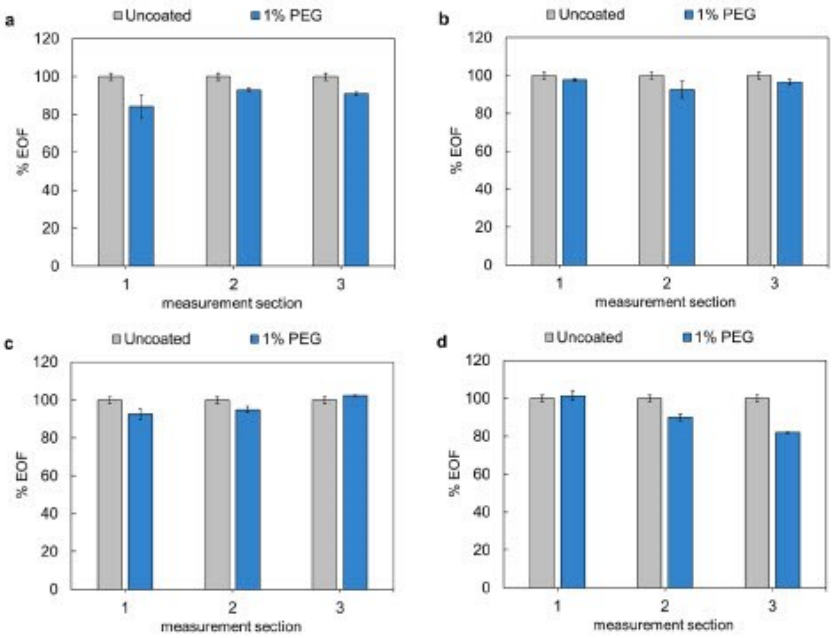


**Fig. 3** Comparison of the EOF suppression and its stability by individual coating methods. Method I (**a**), method II (**b**), method III (**c**), and method IV (**d**) either with 1% (*w/v*) (blue) or 10% (*w/v*) (green) solution of polyethylene glycol. EOF values are presented as medians for each section in comparison to the EOF in an uncoated capillary (grey)

Additionally, the adsorption of this positively charged polymer is enhanced due to electrostatic interactions with negatively charged capillary wall. A relatively stable coating was achieved when using method I (Fig. 4a) for both polymer concentrations and throughout twenty repeated measurements, the EOF for both concentrations reached practically identical values. However, initial deformation of the thiourea peak and subsequent abrupt changes in the EOF were observed for a 10% solution, likely due to partial capillary blockage by the polymer at the beginning.

Repeated flushing by method II (Fig. 4b) resulted in stable suppression of the EOF. On the other hand, larger local fluctuations were observed compared to method I. There was a reoccurring trend where after changing the BGE for a fresh one, a sudden acceleration of the reversed EOF was observed, followed by gradual decreases. As previously, thiourea peak deformation was observed in all obtained electropherograms when using the 10% solution.

For this polymer, a voltage of –30 kV was used instead of + 30 kV as used with previous polymers. No significant changes in stability were observed; however, the reversed EOF was slightly faster with voltage application for method III (Fig. 4c) and almost the same for method IV (Fig. 4d). The effect of voltage for the 10% solution was not tested, as it was evident that the voltage application did not impact the coating when using 1% PDMAC solution, moreover this concentration leads to the deformation of the thiourea peak.

For the 10% solution of PDMAC, the effect of voltage was not tested because the results obtained for the 1% solution indicated that voltage application did not significantly impact the coating.

## Permanent coating with linear polyacrylamide

The permanent coating with linear polyacrylamide was performed according to the procedure used by Hamidli et al. [24] for the electrophoretic separation of proteins. The EOF mobility values were very low so the method by Williams and Vigh [6] was used.

This coating resulted in almost complete elimination of the EOF as all obtained values were between $10^{-11}$ to $10^{-10}$ $m^2\ V^{-1}\ s^{-1}$, suppressing it from one hundred to a thousand times more compared to all tested dynamic coatings. Compared to the uncoated capillary, the EOF was suppressed to 0.40% of the original value. Throughout a sequence of repeated measurements, no significant increase in the EOF was observed, demonstrating the high stability of the coating.

## Liposome sample measurement

In the typical mode of CE operation, the sample is injected at the long end of the capillary, i.e. the end farther from the detection window, and analytes are separated by their electrophoretic migration under an applied voltage. It is

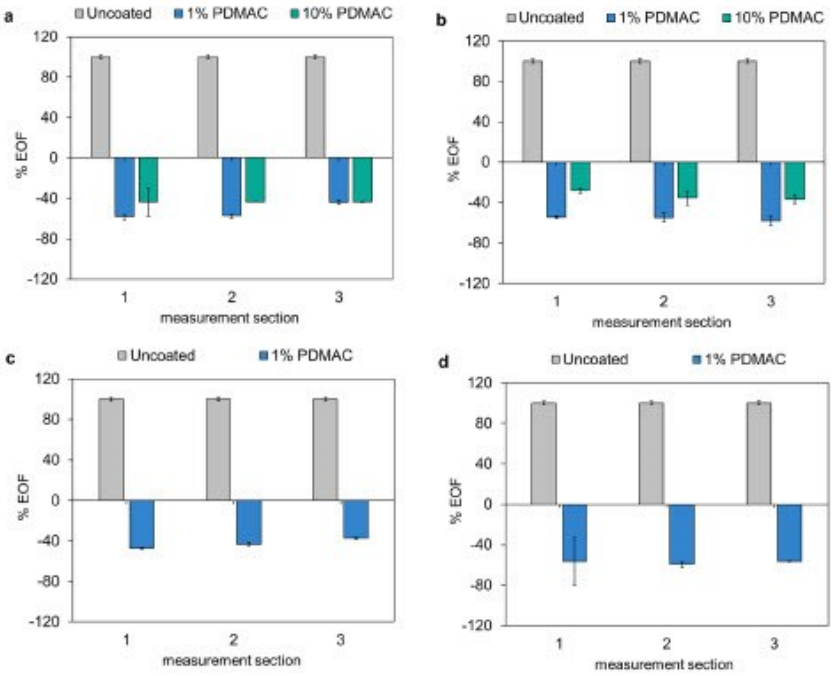


**Fig. 4** Comparison of the EOF suppression and its stability by individual coating methods. Method I (**a**), method II (**b**), method III (**c**), and method IV (**d**) either with 1% (*w/v*) (blue) or 10% (*w/v*) (green) solution of polydiallyldimethylammonium chloride. EOF values are presented as medians for each section in comparison to the EOF in an uncoated capillary (grey)

also possible to inject the sample at the short end of the capillary, leading to faster analysis and reduced possibility of liposome adsorption to the capillary wall as their path through the capillary is shorter. In both of these CE modes, we see the zones in the state in which they pass through the detector at the end of separation and we are not able to observe the development of the zones during the separation process. Phenomena like adsorption on the capillary wall or electromigration dispersion can lead to severe deformations of the analyte zones and corresponding peaks observed. They can even cause such severe dispersion that the zones cannot be detected. In our study, we thus employed a method for studying the separation processes in their earlier stages. The sample was injected into the capillary by the pressure of 5 kPa and moved further in using the same pressure for 30 s. Subsequently, the voltage of 20 kV was applied for different periods and finally, it was moved to the detector by the pressure of 5 kPa. Each measurement was repeated three times for each procedure, i.e., without voltage application or with voltage applied for 10, 20, 30, 50, and 90 s.

Before the description and discussion of the results obtained using uncoated capillary and individual tested coatings, it is necessary to note that the interactions of liposome surface with the capillary wall or specific wall coating can be influenced by the composition of liposomes, the presence of PEG, their surface charge, or pH of BGE. The comparison of coatings we offer is thus not universally valid for all liposomes and conditions but applies to the liposome type used in this work, i.e., PEGylated DPPC-DPPG liposomes with a negative surface charge (− 33 mV), in a BGE consisting of 10 mmol $dm^{-3}$ sodium phosphate buffer at pH = 7.10.

## Uncoated capillary

At first, we used a fused-silica capillary and we observed, that with increased time of applied voltage, the liposome peak initially broadens and eventually disappears (Fig. 5). It was likely caused by the sorption of liposomes onto the capillary wall, thus some modification of the capillary surface was necessary.

Measured samples contained certain unidentified neutral fluorescent compounds that we used as an EOF marker for LIF detection. The mobility of this zone was matched with the mobility of the thiourea zone observed by the UV detector, which confirmed that the fluorescent compounds in question lack their own electrophoretic migration. As can be seen from Figs. 5, 6, 7, the EOF peak was absent with increased time of applied voltage, suggesting that it migrated out of the inlet capillary end during the voltage application period before the final pressure was applied.

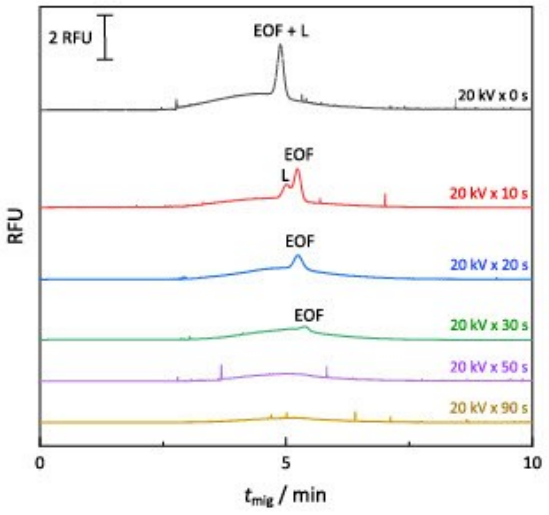


Fig. 5 CE-LIF measurement of the fluorescently labeled liposome (L) with increasing time of applied voltage in an uncoated capillary

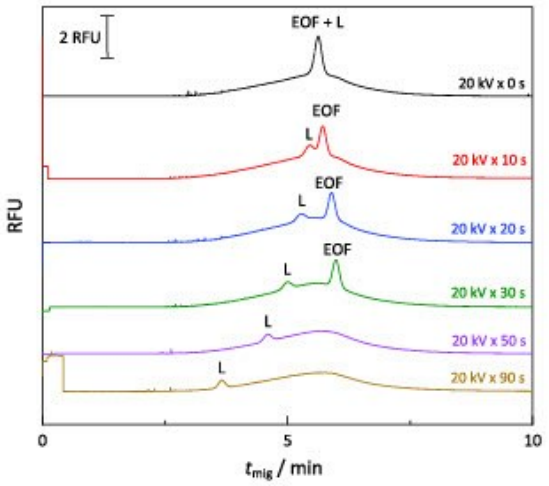


Fig. 6 CE-LIF measurement of the fluorescently labeled liposome (L) with increasing time of applied voltage in a capillary permanently coated with PVA

## Commercially coated capillary with polyvinyl alcohol

To overcome the problem with the adsorption, we used a capillary that was commercially coated with polyvinyl alcohol (PVA) and where the EOF was fully suppressed. During our experiments, we observed a shift in the peak of liposomes towards shorter times with an increased time of applied voltage (Fig. 6). This migration behavior proves

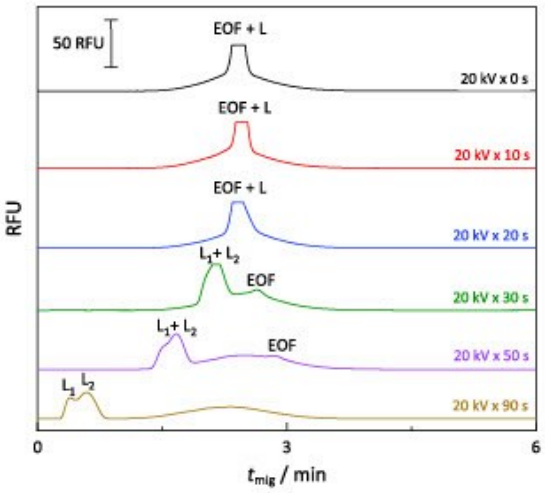


Fig. 7 CE-LIF measurement of the fluorescently labeled liposome ($L_1$ and $L_2$) with increasing time of applied voltage in a capillary dynamically coated with the 10% PVP

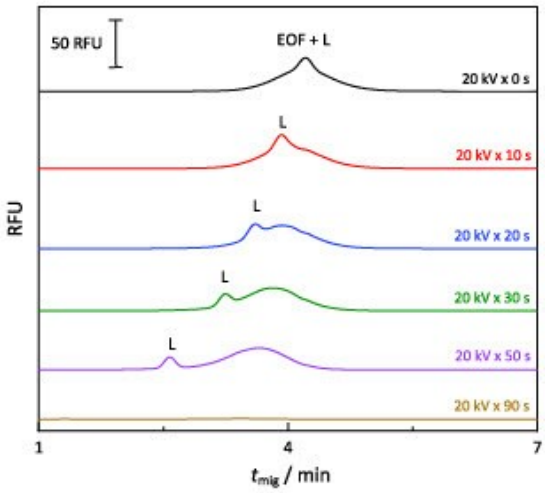


Fig. 8 CE-LIF measurement of the fluorescently labeled liposome (L) with increasing time of applied voltage in a capillary permanently coated with the LPA

that they are negatively charged as they migrate toward the anode, which is opposite to the EOF direction. In this case, the EOF was directed away from the detector, causing a gradual increase in the neutral zone time. Once again, when the voltage was applied for too long, the marker exited the capillary at the injection end and thus was not detected by the detector.

Additionally, the liposome peak maintained its shape and size compared to previous measurements conducted in the uncoated capillary, where the liposome peaks disappeared due to adsorption.

While the use of commercially coated capillary proved effective in minimizing the sorption-related issues, their financial demands prompted us to explore the development of an effective and cost-effective method of liposome adsorption elimination. Our objective was to establish a stable methodology with suppressed EOF, enabling the accurate analysis of liposomes without the use of commercially coated capillaries.

### Dynamically coated capillary with 10% PVP

The separation in the capillary coated with the 10% solution of PVP using method I provided peaks that were not significantly broadened or deformed. Electropherogram (Fig. 7) shows, that liposome peak again shifted towards lower migration times, which is consistent with their negative charge. However, a partial peak splitting was observed after applying the voltage for longer times. This suggests that this freshly prepared sample of liposomes might have been less homogenous, containing more than one liposomal fraction. Additionally, it has a higher signal intensity which can be caused due to an error during preparation as probably a higher amount of fluorescently labeled lipid was used. Despite this, the overall separation in the PVP-coated capillary appeared to be efficient, with minimal peak distortion, indicating the potential suitability of this coating for stable and reliable liposome analysis in capillary electrophoresis.

### Permanently coated capillary with LPA

In the capillary permanently coated with LPA (Fig. 8), the EOF was effectively suppressed, eliminating any potential error in the mobility measurements of liposomes. As previously, we observed a gradual decrease in the migration times of liposomes with increasing time of applied voltage. Notably, the peak of the liposome appeared more symmetrical compared to previous coatings. This suggested that the LPA coating effectively minimized any potential interactions that could lead to peak broadening or distortion. Overall, the use of a permanent LPA coating demonstrated promising results in accurate and reliable analysis of liposomes.

## Conclusion

The effectiveness of EOF suppression and stability of its mobility were evaluated for various types of tested coatings where thiourea was used as an EOF marker. For dynamic coatings, four coating methods were employed with four coating agents. The results varied depending on the method and concentration of the polymer used. Pluronic F-127

coatings exhibited limited stability, with varying degrees of EOF suppression observed across different methods and polymer concentrations. Polyvinyl pyrrolidone K30 coatings showed relatively stable performance, particularly with a 10% solution, but exhibited gradual EOF reduction over time. Polyethylene glycol coatings did not effectively suppress EOF, and the stability of the coating was poor across all methods tested. Polydiallyldimethylammonium chloride coatings provided stable EOF suppression, with a simple one-time flush method demonstrating consistent performance for both concentrations. For permanent coating, linear polyacrylamide was tested and it effectively eliminated the EOF, where the suppression was one hundred to a thousand times greater compared to all tested dynamic coatings. This coating demonstrated high stability throughout repeated measurements.

Additionally, liposome sample measurements with the LIF detection method were conducted using different capillaries: uncoated, commercially coated, dynamically coated, and permanently coated capillary. Commercial polyvinyl alcohol-coated capillary allowed us to analyze liposomes in a special CE mode. Dynamically coated capillary with 10% polyvinyl pyrrolidone K30 provided stable separations, peak splitting was observed with prolonged voltage application due to the polydispersity of the sample. When using permanently coated capillaries, we were able to measure under completely suppressed EOF as in the case of LPA-coated capillary. The peaks were symmetrical, indicating minimal interaction between the capillary wall and liposomes.

Overall, the study demonstrates the potential of various coating methods for EOF suppression in capillary electrophoresis, with each method offering unique advantages and considerations for optimal analysis of liposomes.

## Experimental

3-(Trimethoxysilyl)propyl methacrylate, ≥ 97%; acetone, ≥ 99.8%; acrylamide, ≥ 99%; *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ≥ 99.5%; ammonium persulfate, 98%; Pluronic F-127; polydiallyldimethylammonium chloride, 20% w/w; polyvinylpyrrolidone K30, special grade and thiourea, 99% were purchased from Sigma-Aldrich (Burlington, USA). Acetonitrile, ≥ 99.9% and hydrochloric acid, 37% w/w, reag. Ph. Eur. were purchased from VWR Chemicals (Radnor, USA). Sodium dihydrogenphosphate dihydrate p.a. was purchased from Lach-Ner (Neratovice, Czech Republic). Sodium hydrogenphosphate dodecahydrate p.a. and polyethylene glycol, $M_r$ 6000 were purchased from Lachema (Brno, Czech Republic). Sodium hydroxide was purchased from Penta (Prague, Czech Republic).

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1–2- distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt (DSPG), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] ammonium salt (DMPE-2000 PEG), and 1-oleoyl-2-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, USA). Those lipids were used to prepare liposomes of total lipid concentration 5 mg $cm^{-3}$ by lipid film hydration method. The lipid composition for liposome preparation was DPPC:DPPG:DMPE-2000 PEG:NBD-PC 75:22:3:0.5. The lipid mixture was dissolved in a chloroform:methanol mixture 2:1 (*v*/*v*). The solvents were evaporated in a rotary evaporator at a constant temperature of 55 °C, gradually lowering pressure from atmospheric to 150 mbar. The dried lipid film was kept in a desiccator for 24 h. Then, the lipid film was rehydrated by a 10 mmol $dm^{-3}$ sodium phosphate buffer at pH = 7.10 and extruded 21 times through a 400 nm membrane. Zetasizer Nanoseries (Malvern Instruments, Worcestershire, UK) was used to characterize the prepared liposomes. We used 2 samples of liposomes of the same composition and they were extruded through a 400 nm membrane, the zeta-potential was in both cases − 33 mV and the mean size of the first one was 319.7 nm, and of the second one 276.4 nm, both had polydispersity index of 0.3. The liposome samples were injected to CE without any further dilution.

All samples and BGE were prepared using deionized water produced by the Milli-Q system from Millipore (Danvers, USA). Fused-silica capillary was purchased from Polymicro Technologies (Phoenix, USA), and polyvinyl alcohol-coated capillary from Agilent Technologies (Waldbronn, Germany). Agilent 7100 CE from Agilent Technologies (Waldbronn, Germany) was used for all electrophoretic measurements, it was equipped with a diode array detector (UV–VIS) and with Zetalif LED (LIF) detector with a fixed excitation wavelength of 480 nm from Picometrics (Labege, France).

### Capillaries

Fused-silica capillary, 50 µm ID, 375 µm OD, 33.0 cm total length, 8.5 cm (UV–Vis detection), and 21.0 cm (LIF detection) effective length was used for measuring in an un-coated capillary. Capillaries of the same properties were used for dynamic coating.

Capillary permanently coated with LPA according to Hamidli et al. [24], 50 µm ID, 375 µm OD, 50.0 cm total length, 8.5 cm (UV–Vis detection), and 21.0 cm (LIF detection) effective length was prepared for measuring under suppressed EOF. The capillary coating procedure was as follows. Firstly, the new fused-silica capillary was flushed with acetone (3 min, 600 kPa), 1 mol $dm^{-3}$ sodium hydroxide (30 min at 60 °C, 500 kPa), deionized water (1 min,

600 kPa), 1 mol $dm^{-3}$ hydrochloric acid (30 min, 500 kPa), and deionized water (1 min, 600 kPa). The capillary was then dried with nitrogen (30 min, 600 kPa; 60 min, 200 kPa). Subsequently, the capillary was flushed with a 1:1 mixture of acetone and 3-(trimethoxysilyl)propyl methacrylate (5 min, 300 kPa) and left overnight with both ends immersed in vials containing this mixture. The following day, the capillary was flushed with acetone (1 min, 200 kPa), dried with nitrogen (60 min, 200 kPa) and flushed with the polymerization agent (4 min at 50 °C, 500 kPa). This agent consisted of 1 $cm^3$ of 4% acrylamide solution, 1 $mm^3$ of *N,N,N′,N′*-tetramethylethylenediamine, and 10 $mm^3$ of 10% ammonium persulfate solution. It was then left with both ends immersed in vials containing this polymerization agent, first for 60 min at 50 °C and then overnight at room temperature. The following day, the excess polymerization agent was removed by flushing the capillary with deionized water (30 min, 600 kPa; 20 min, 800 kPa).

Commercially coated capillary with polyvinyl alcohol, 50 μm ID, 64.5 cm total length, 8.5 cm (UV–Vis detection), and 21.0 cm (LIF detection) effective length was used for measuring under suppressed EOF.

### EOF determination

All electrophoretic experiments in this article were performed in a 10 mmol $dm^{-3}$ sodium phosphate buffer, pH = 7.10. The buffer was prepared by weighing appropriate amounts of sodium dihydrogenphosphate dihydrate and sodium hydrogenphosphate dodecahydrate, dissolving them, and diluting them with deionized water so that each of the components had a final concentration of 5 mmol $dm^{-3}$.

Measurements of EOF in dynamically coated capillaries were conducted by hydrodynamically injecting 0.1 mg $cm^{-3}$ aqueous solution of thiourea by a pressure of 5 kPa for 5 s. After the sample injection, a separation voltage of 20 kV was applied to the capillary. Except for the PDMAC coating agent, for which a negative voltage of −20 kV was applied.

EOF measurements in permanently coated capillary were performed as follows: the first thiourea zone was injected hydrodynamically by a pressure of 5 kPa for 5 s and then mobilized with a pressure of 10 kPa for 60 s. The same procedure was applied for the injection and mobilization of the second thiourea zone, followed by the application of a voltage of 20 kV to the capillary for 120 s. The third thiourea zone was injected again for 5 s by a pressure of 5 kPa. After this injection sequence, the zones in the capillary were mobilized by applying a pressure of 10 kPa at the inlet end of the capillary until all three thiourea zones passed through the detector.

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**Data availability** Raw data are available upon request from the corresponding author.

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PUBLICATION III (MANUSCRIPT)

# Investigating Drug-Liposome Interactions Using Liposomal Electrokinetic Chromatography

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**Abbreviations**

**API**, active pharmaceutical ingredient; **BGE**, background electrolyte; **CE**, capillary electrophoresis; **CEC**, capillary electrochromatography; **DPPC**, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; **DPPG**, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt); **EOF**, electroosmotic flow; **LEKC**, liposomal electrokinetic chromatography

**Keywords**:

Active pharmaceutical ingredients/Capillary electrophoresis/Interactions/Liposomal electrokinetic chromatography/Liposomes/Pseudostationary phase

**Abstract**

This study explores the potential of using liposomes as a pseudostationary phase in capillary electrophoresis to examine drug-lipid interactions, which are crucial for enhancing drug delivery systems. We observed that the increasing amount of liposomes in the background electrolyte significantly affected the separation kinetics of various active pharmaceutical ingredients, altering their mobility and/or peak shapes. Experiments with liposomes from bovine liver and heart tissue extracts revealed different interactions based on the lipid composition. Canagliflozin, which initially showed no electrophoretic mobility, migrated toward the anode in the presence of negatively charged liposomes. Mobility of positively charged substances, ambroxol and maraviroc, was suppressed by the interactions with liposomes. Their peaks also exhibited significant tailing. The effect on the separation of negatively charged compounds was significantly weaker. A small change in mobility was observed only in the case of deferasirox. We also examined the effect of temperature during separation, and we observed that increased temperature generally enhanced effective mobility due to lower electrolyte viscosity and increased lipid bilayer fluidity. Lastly, we tested the effect of sodium phosphate buffer pH (ranging from 6.0 to 8.0) with 4% liposomes on drug-liposome interactions. However, the effects were complex due to changes in API ionization and liposome surface charge, complicating the distinction between pH effects and liposome presence on API behavior. Our findings emphasize the significance of liposome composition, temperature, and pH in studying the interactions of liposomes with drugs, which is crucial for optimizing liposome-based drug delivery systems.

**Introduction**

In recent years, the study of drug-lipid interactions has gained significant attention in pharmaceutical research, driven by the growing recognition of lipid-based drug delivery systems as a promising tool for enhancing drug solubility, bioavailability, and targeting [1,2]. Among these systems, liposomes stand out as versatile nanoparticles that are capable of encapsulating a wide range of substances, therefore they may serve as a promising carrier for drug delivery and targeted therapy. Moreover, their biocompatibility and easily modifiable physicochemical properties make them an attractive focus for research [3,4].

Studying the affinity of active pharmaceutical ingredients (APIs) to liposomes plays an essential role in determining whether liposomes can effectively be employed as carriers for APIs or not [5]. The use of liposomes to simulate biological membranes allows us to study how individual APIs interact with the lipids on the membrane's surface or how they may penetrate it. This understanding is essential for understanding the mechanisms of drug passage through those membranes, which is crucial for pharmaceutical research [6,7]. The transport of pharmaceutical compounds is a critical aspect of early drug development and one of the most common mechanisms is passive diffusion. It refers to the movement of substances across biological membranes driven by concentration gradients. When evaluating the suitability of APIs for passive diffusion, several characteristics come into play [8].

Lipophilic compounds are more likely to pass through biological membranes. The lipophilicity is often quantified using parameters such as partition coefficient (log P) and distribution coefficient (log D). While lipophilicity is essential, drugs should also be sufficiently soluble in water to ensure their easy absorption into the bloodstream and effective distribution throughout the body. Lipophilic and water-soluble compounds are more likely to be membrane-permeable, which is crucial for reaching targeted sites within the body [9-11]. The evaluation of permeability through membranes can be done by several methods and capillary electrophoresis is one of them. Either as capillary electrochromatography (CEC) or as liposomal electrokinetic chromatography (LEKC) [12].

Liposomal electrokinetic chromatography combines elements from both capillary electrophoresis and chromatography. In this approach, liposomes are freely suspended within the background electrolyte (BGE) and they serve as a pseudostationary phase inside the capillary, facilitating chromatographic separation. Through a combination of electrophoretic mobility, electroosmotic flow, and chromatographic interactions with the liposomal phase, LEKC enables the study of API-liposome interactions under controlled electrophoretic conditions. Understanding these interactions is crucial for optimizing drug delivery systems, for unraveling drug release mechanisms, and for predicting in vivo behavior [12-14]. Such interactions can influence the kinetics of analytical separations in various ways, including changes in mobilities or alterations in peak shapes. The utilization of CE with liposomes as a pseudostationary phase offers several advantages, such as enhanced separation efficiency and the ability to closely mimic the physiological conditions of biological membranes by varying the lipid composition. This approach not only deepens our understanding of drug-lipid interactions but also supports the development of more effective and targeted drug delivery systems.

Franzen et al. [15] used CE for physicochemical characterization of polyethylenglycolated liposomes with encapsulated oxaliplatin, they compared the electrophoretic mobilities determined by CE with laser Doppler electrophoresis results. Moreover, they compared the average hydrodynamic diameters of prepared liposomes determined by Taylor dispersion analysis with data obtained from dynamic light scattering. Lastly, they studied the interactions between liposomes and oxaliplatin, propranolol, and paracetamol by LEKC and CE-frontal analysis. Interaction studies with propranolol

indicated successful determination of distribution coefficients, highlighting CE's utility in characterizing nanoparticulate drug formulations.

Manetto et al. [16] studied the interactions of various drugs of different polarities and chemical structures with liposomes prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in a polyimide-coated capillary with suppressed electroosmotic flow (EOF). They used 50 mmol/l phosphate buffers at pH 7.5 and 9.2. At pH 7.5, drugs exhibited partial binding with the liposomes, while at pH 9.2, the interaction was stronger, leading to complete binding.

Wang et al. [17] explored the potential of LEKC as a method for predicting the skin permeability of 23 compounds. They used liposomes composed of phosphatidylcholine and phosphatidylserine in a 4:1 molar ratio containing 6 mM cholesterol. They found a strong correlation ($R^2 = 0.902$) between the compound's skin permeability coefficient (log $K_p$) and retention factor values (log k). LEKC demonstrated more similarity to skin penetration compared to commonly used octanol-water partitioning, suggesting it as a promising tool for predicting transdermal drug delivery.

In this work, we study the importance of several experimental parameters, namely lipid composition of liposomes, temperature, and pH, for reliable application of LEKC in the assessment of drug-membrane interactions. The impact of drug-liposome interactions on the effective mobility and peak shape is investigated for a set of positive, neutral, and negatively charged APIs, comparing the results obtained with three different types of liposomes composed of (i) dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol, (ii) bovine liver polar lipid extract, and (iii) bovine heart polar lipid extract.

## Material and methods

### *Chemicals and reagents*

Thiourea, 99% was purchased from Sigma-Aldrich (Burlington, USA). Sodium dihydrogen phosphate dihydrate, p.a. was purchased from Lach-Ner (Neratovice, Czech Republic). Sodium hydrogen phosphate dodecahydrate, p.a. was purchased from Lachema (Brno, Czech Republic). Sodium hydroxide was purchased from Penta (Prague, Czech Republic). All standards of active pharmaceutical ingredients namely ibuprofen, valsartan, febuxostat, canagliflozin, atorvastatin calcium, maraviroc, deferasirox, aprepitant, and ambroxol hydrochloride were provided by Zentiva (Prague, Czech Republic). Methanol, ≥99% was purchased from VWR International (Radnor, USA). All samples and background electrolytes were prepared using deionized water produced by the Milli-Q system from Millipore (Danvers, USA).

Lipids for preparation of liposomes, namely 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DPPG); Liver polar lipid extract (bovine) and Heart polar lipid extract (bovine) were purchased from Avanti Polar Lipids (Alabaster, USA). DPPC and DPPG were mixed in the 3:1 molar ratio. The composition of liver and heart polar extracts is shown in **Table 1**. All samples were prepared with a total lipid concentration of 5 mg/ml by lipid film hydration method [18] in 10 mmol/l sodium phosphate buffer at pH 7.10. Prepared liposomes were negatively charged and were extruded through a membrane with a pore size of 400 nm.

*Instrumentation*

All experiments were performed on a 7100 CE instrument from Agilent Technologies (Waldbronn, Germany) equipped with a diode array detector. A fused-silica capillary was purchased from Polymicro Technologies (Phoenix, USA). For the measurement of pH, a 3540 pH/conductivity meter from Jenway (Staffordshire, UK) was used.

*Electrophoretic conditions*

The inner diameter of the fused-silica capillary was 50 μm, the outer diameter was 375 μm, the total length was 50.0 cm and the effective length was 41.5 cm. The capillary temperature was maintained at 25 °C unless stated otherwise. Before every set of measurements, the capillary was flushed for 10 minutes with 1 M sodium hydroxide and 10 minutes with deionized water. Before each run, the capillary was flushed for 2 minutes with the background electrolyte. Samples were injected hydrodynamically by a pressure of 5 kPa for 5 s. A separation voltage of 20 kV was then applied, and the electric current in 10 mM sodium phosphate buffer at pH 7.10 without the addition of liposomes was approximately 17 μA. UV detection at 200 nm was employed in all cases.

**Results and discussion**

*Preliminary experiments*

For the preliminary experiments, we used liposomes prepared from DPPC and DPPG lipids in a 3:1 molar ratio hydrated in 10 mM sodium phosphate buffer, pH 7.10. At first, four APIs were selected to test the effect of the liposome amount in the BGE on the separation kinetics (**Fig. 1**). We used two APIs that have a positive charge (maraviroc and ambroxol hydrochloride), one neutral (canagliflozin) and one with a negative charge (deferasirox) under selected conditions. Three of four APIs showed changes either in their mobility or peak shape with increasing amount of liposomes added to BGE (**Table 2**). Originally, canagliflozin (peak no. 3) did not exhibit electrophoretic mobility in the phosphate buffer without the presence of liposomes. When they were added into the BGE, canagliflozin migrated out of the neutral zone in the anodic direction. Its peak broadened with the increasing amount of liposomes keeping a symmetrical shape so the USP tailing factor did not change significantly while its anodic effective mobility linearly increased. Positively charged maraviroc (peak no. 2) showed small changes in the peak shape as the USP tailing factor at 5% height increased about 1.5 times indicating occurring interactions with liposomes. Tailing of the peak, increasing with the increasing amount of liposomes, could point out to a slow kinetics of the interaction, on the other hand, its effective mobility remained unchanged. This would suggest that due to the slow interaction kinetics only a small fraction of maraviroc molecules engaged in the interaction, which led to increased tailing but unchanged overall effective mobility. The second positively charged API, ambroxol hydrochloride (peak no. 1), showed a substantial change in the peak shape and also a significant decrease in its cathodic effective mobility. As little as 2% (*v/v*) of liposomes in the BGE increased the USP tailing factor at 5% height more than 2 times. With increasing amount of liposomes the peak broadened even more and with decreasing effective mobility it fully disappeared in the baseline. The dramatic change in the peak shape and significant tailing points to a relatively slow kinetics of the interaction. Unlike maraviroc, the effective mobility of ambroxol decreased to less than 80% of its original value in 6% (*v*/*v*) liposomes indicating a significant part of ambroxol molecules engaging in the interaction. Interestingly, the log P values (see **Table 3**) suggest higher lipophilicity of maraviroc when compared to ambroxol. The higher lipophilicity of maraviroc might hinder the electrostatic interactions between negatively charged liposomes and positively charged API. On the other hand, the negatively charged deferasirox (peak no. 4) showed no changes in peak shape or/and effective mobility. This finding supports the importance of electrostatic

interactions between API and liposomes. Nevertheless, it should be noted that due to the anodic effective mobility of deferasirox and the anodic effective mobility of liposomes an interaction of the same strength will result in a significantly smaller change in the effective mobility than in the case of neutral or positive API. Overall, the preliminary experiments showed that in this experimental setup, it is possible to observe interactions of the APIs with liposomes, which can manifest as changes in mobility as well as changes in peak shape and thus we proceeded to experiments with more biologically relevant liposomes based on tissue extract lipids.

*Experiments with liposomes from tissue extracts*

The same experiment was performed with liposomes prepared from tissue extracts, either from bovine liver (**Fig. 2a**) or from bovine heart (**Fig. 2b**). As previously, canagliflozin (peak no. 3) migrated out of the neutral zone; however, its anodic effective mobility was in both cases roughly one order of magnitude higher than in the case of DPPC-DPPG liposomes, with a more pronounced increase in the liver-based liposomes (**Table 4** and **5**). An interesting difference was observed also in peak symmetry. While in the DPPC-DPPG liposomes, the canagliflozin peak was relatively symmetrical (USP tailing 1.01 to 1.24), it was fronting in the BGE containing liver extract liposomes (USP tailing 0.68 to 0.91). Surprisingly, the peak was getting more symmetrical with the increasing amount of liposomes. On the other hand, in BGE with heart extract liposomes canagliflozin peak was tailing without a significant difference in the USP tailing factor value (1.93 to 2.00). From **Fig. 2b** it can be seen that although the symmetry was roughly constant peak was broadening with the increasing amount of liposomes. Both tissue extract-based liposomes thus exhibited significantly stronger interactions with canagliflozin, which gained considerably higher anodic mobility. Differences between the lipid composition of both extracts (**Table 1**) caused liver extract liposomes to influence the mobility stronger than the heart extract liposomes. This difference was strongly manifested by the canagliflozin peak fronting in the BGE with liver extract liposomes and tailing in the BGE with heart extract liposomes. A striking difference in the interactions with liposomes from heart and liver extracts was observed for maraviroc (peak no. 2). Similarly to the DPPC-DPPG liposomes, the maraviroc peak was tailing and the USP tailing factor was increasing with the increasing amount of liver/heart extract liposomes in BGE. Concerning the cathodic effective mobility of this API, both extract-based liposomes showed a stronger impact on the mobility than DPPC-DPPG liposomes. Going from 0 to 10 % (*v*/*v*) of liposomes in BGE, the effective mobility decreased to 90% of its original value with heart extract liposomes and to roughly 20% with liver extract liposomes. Ambroxol hydrochloride (peak no. 1) lost its cathodic mobility when liver-based liposomes were added to the BGE, indicating strong interactions with these liposomes. A similar observation was made with heart-based liposomes, although a higher liposome concentration in the BGE was needed for the API to lose its mobility. In heart-based liposomes, the USP tailing factor increased 4.1 times with the addition of only 2% (*v/v*) liposomes to the BGE. For deferasirox (peak no. 4), once again the presence of liposomes had no distinct effect on tailing factor values regardless of whether they were prepared from liver or heart extract. Anodic effective mobility of this negatively charged API slightly increased (by around 6%) in the presence of liver extract liposomes, an even smaller increase (roughly 2.5%) was observed for heart extract liposomes. As discussed above, the weak effect of liposomes on the mobility of negatively charged deferasirox can be caused either by its weak interaction with liposomes possibly hindered by their electrostatic repulsion, or by the anodic effective mobility of both, API and liposomes. However, when deferasirox was measured without the presence of other APIs, its peak shape changed from fronting to tailing, suggesting that the liposomes might be saturated by other APIs in the mixture. These results suggest that the interactions between APIs and liposomes are strongly influenced by the lipid composition and charge of the liposomes. Liver-based liposomes, with their higher content of negatively charged components, exhibit stronger electrostatic interactions with certain APIs, leading to more significant changes in mobility and tailing

factors. The differences observed in maraviroc's behavior indicate that the specific lipid components of liver and heart extracts affect its interaction dynamics. The saturation of liposomes by other APIs in the mixture can also impact the observed peak shapes and mobilities, as seen with Deferasirox. Overall, these findings highlight the importance of considering lipid composition and thus their different properties when evaluating API-liposome interactions in CE experiments.

*Effect of temperature*

We tested the influence of increasing temperature on API-liposome interactions for nine lipophilic APIs (**Fig. 3**), namely ambroxol hydrochloride, maraviroc, canagliflozin, deferasirox, aprepitant, atorvastatin calcium, febuxostat, ibuprofen, and valsartan. As the temperature rises, the flowability of the lipid bilayer increases, which should affect these interactions. Based on previous experiments, we added 4% liposomes to a 10 mM sodium phosphate buffer at pH 7.10. We observed that the effective mobility increased linearly with temperature for most APIs (**Fig. 4**), which can be attributed not only to the lower viscosity of the BGE, but also to the enhanced fluidity of the liposomal membrane. This makes it easier for the APIs to migrate through the capillary. However, canagliflozin (peak no. 3) exhibited different behavior. In liver-based liposomes, its anodic effective mobility remained almost unchanged, while in heart-based liposomes, its effective mobility decreased, in contrast to all other tested APIs. This suggests that canagliflozin has a lower affinity for liposomes as the lipid bilayer becomes less rigid with increasing temperature. The unique behavior of canagliflozin indicates that its interaction with liposomes is particularly sensitive to changes in the lipid bilayer's rigidity as a significant reduction in interaction strength can be observed at higher temperatures. To compare the behavior of canagliflozin with another uncharged API, we added aprepitant (API no. 5) into the mixture. However, the aprepitant exhibited zero electrophoretic mobility throughout all our experiments, suggesting no ongoing interactions with our liposomes. Overall, this experiment highlights the importance of considering temperature effects when evaluating API-liposome interactions as these effects can significantly differ for individual APIs. The differences in behavior between liver-based and heart-based liposomes also underscore the role of lipid composition in these interactions. Understanding these variables can help in the optimization of liposome-based drug delivery systems, ensuring effective drug encapsulation and release under physiological conditions.

*Effect of pH*

Lastly, we tested how the different pH levels might influence API-liposome interactions, considering that some APIs might exhibit pH sensitivity. We prepared six sodium phosphate buffers with an ionic strength of 20 mmol/l, with pH values ranging from 6.0 to 8.0 in 0.5 increments, and measured the interactions in BGEs containing 4% liposomes (**Fig. 5**). It turned out to be challenging to distinguish whether the observed changes in API behavior were due to the variations in API charge at different pH levels or if the presence of liposomes also contributed to these changes. The influence of pH on API-liposome interactions is a complex phenomenon, as both the charge of the APIs and the liposomal membrane can be affected by pH changes. APIs can ionize differently at various pH levels, altering their charge and subsequently their interaction with the liposomes. Similarly, the surface charge of the liposomes can change with pH, influencing the electrostatic interactions between the APIs and the liposomal membrane. In our experiments, the difficulty in distinguishing the effects of pH changes from the presence of liposomes suggests that both factors likely play significant roles in modifying API behavior.

**Conclusion**

We explored the interactions between various APIs and liposomes used as a pseudostationary phase in liposomal electrokinetic chromatography. Our preliminary experiments demonstrated that liposomes prepared from DPPC and DPPG lipids can influence the separation behavior of APIs. Experiments with liposomes derived from bovine liver and heart tissue extracts revealed distinct interaction patterns. Effective mobilities of neutral canagliflozin, positive maraviroc, and ambroxol as well as negative deferasirox showed stronger interactions with liver-based liposomes, attributed to their higher negative charge and different lipid composition. Changes in peak shapes displayed varied interaction behaviors between liver-based and heart-based liposomes, highlighting the role of lipid composition in API-liposome interactions. Notably, deferasirox's peak shape changed when measured alone, suggesting potential saturation effects in the presence of other APIs. Temperature studies highlighted that increasing the temperature generally enhanced the effective mobility of most APIs, due to lower BGE viscosity and increased liposomal membrane fluidity. However, canagliflozin's mobility decreased at higher temperatures, particularly in heart-based liposomes, indicating a reduced affinity as the lipid bilayer became less rigid. This suggests that canagliflozin's interaction with liposomes is sensitive to changes in the bilayer's rigidity. Lastly, pH studies revealed the complexity of the effects of pH and liposome presence on API behavior. Changes in API charge at different pH levels and the corresponding alterations in liposomal surface charge both play significant roles in API-liposome interactions. Overall, our findings underscore the importance of considering lipid composition, temperature, and pH when evaluating API-liposome interactions in CE experiments. These factors significantly impact the behavior of APIs and can influence the optimization of liposome-based drug delivery systems for enhanced drug encapsulation, release, and targeting under physiological conditions.

**Declarations**

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Compliance with Ethical Standards

This study does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

Consent for publication

Authors agree with the publication of the manuscript.

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**Table 1** – Lipid composition of individual extracts [19], PC – phosphatidylcholine, PE – phosphatidylethanolamine, PI – phosphatidylinositol, PA – phosphatidic acid, CA – cardiolipin

| Heart extract (bovine) | | Liver extract (bovine) | |
|---|---|---|---|
| Component | wt/wt% | Component | wt/wt% |
| PC | 8.6 | PC | 42 |
| PE | 13.6 | PE | 26 |
| PI | 1.0 | PI | 9 |
| PA | 0.6 | Lyso PI | 1 |
| CA | 1.7 | Cholesterol | 5 |
| Neutral lipid | 57.7 | Others (neutral lipids) | 17 |
| Unknown | 16.8 | | |

**Table 2** – Obtained electrophoretic mobilities $\mu_{eff}$ and USP tailing factor in BGE containing liposomes from DPPC and DPPG at total lipid concentration 5 mg/ml in 3:1 molar ratio; BGE: 10 mM sodium phosphate buffer with increasing amount of liposomes

| | $\mu_{eff} \cdot 10^8$, $m^2\ V^{-1}\ s^{-1}$ | | | | USP tailing (at 5% height) | | | |
|---|---|---|---|---|---|---|---|---|
| % lip | Ambroxol | Maraviroc | Canagliflozin | Deferasirox | Ambroxol | Maraviroc | Canagliflozin | Deferasirox |
| 0 | 1.57 ± 0.02 | 0.95 ± 0.02 | - | -1.73 ± 0.02 | 2.96 | 3.77 | - | 0.57 |
| 2 | 1.52 ± 0.01 | 1.01 ± 0.01 | -0.12 ± 0.00 | -1.71 ± 0.01 | 6.77 | 3.98 | ND* | 0.56 |
| 4 | 1.41 ± 0.01 | 1.01 ± 0.02 | -0.21 ± 0.00 | -1.72 ± 0.01 | ND* | 4.13 | 1.24 | 0.56 |
| 6 | 1.23 ± 0.03 | 1.00 ± 0.01 | -0.29 ± 0.01 | -1.72 ± 0.00 | ND* | 4.13 | 1.01 | 0.56 |
| 8 | - | 0.99 ± 0.01 | -0.38 ± 0.01 | -1.71 ± 0.00 | - | 4.96 | 1.03 | 0.57 |
| 10 | - | 0.99 ± 0.02 | -0.45 ± 0.01 | -1.72 ± 0.00 | - | 5.21 | 1.12 | 0.58 |

ND* – not determined due to a partial overlap with another peak

**Table 3** – Used APIs and their characteristics

| No. | API | *pKa* [20] | log *P* [20] | charge at pH 7.10 |
|---|---|---|---|---|
| **1** | Ambroxol HCl | 15.26 | 2.65 | + |
| **2** | Maraviroc | 13.98 | 3.63 | + |
| **3** | Canagliflozin | 12.57 | 3.52 | n |
| **4** | Deferasirox | 4.51 | 4.74 | - |
| **5** | Aprepitant | 6.59 | 5.22 | n |
| **6** | Atorvastatin Ca | 4.31 | 5.39 | - |
| **7** | Febuxostat | 3.08 | 3.52 | - |
| **8** | Ibuprofen | 4.85 | 3.84 | - |
| **9** | Valsartan | 4.35 | 5.27 | - |

**Table 4** – Obtained electrophoretic mobilities $\mu_{eff}$ and USP tailing factor in BGE containing liposomes from liver extracts at total lipid concentration 5 mg/ml; BGE: 10 mM sodium phosphate buffer with increasing amount of liposomes

| | $\mu_{eff} \cdot 10^8$, $m^2\ V^{-1}\ s^{-1}$ | | | | USP tailing (at 5% height) | | | |
|---|---|---|---|---|---|---|---|---|
| % lip | Ambroxol | Maraviroc | Canagliflozin | Deferasirox | Ambroxol | Maraviroc | Canagliflozin | Deferasirox |
| 0 | 1.56 ± 0.02 | 0.95 ± 0.01 | - | -1.70 ± 0.02 | 2.97 | 3.19 | - | 0.64 |
| 2 | - | 0.83 ± 0.01 | -1.78 ± 0.00 | -1.70 ± 0.01 | - | 3.52 | ND* | 0.68 |
| 4 | - | 0.62 ± 0.01 | -2.51 ± 0.01 | -1.73 ± 0.00 | - | ND* | 0.68 | 0.59 |
| 6 | - | 0.49 ± 0.01 | -2.83 ± 0.01 | -1.75 ± 0.00 | - | ND* | 0.76 | 0.58 |
| 8 | - | 0.31 ± 0.00 | -3.12 ± 0.01 | -1.79 ± 0.00 | - | ND* | 0.84 | 0.61 |
| 10 | - | 0.17 ± 0.02 | -3.30 ± 0.03 | -1.81 ± 0.01 | - | ND* | 0.91 | 0.67 |

ND* – not determined due to a partial overlap with another peak

**Table 5** – Obtained electrophoretic mobilities $\mu_{eff}$ and USP tailing factor in BGE containing liposomes from heart extracts at total lipid concentration 5 mg/ml; BGE: 10 mM sodium phosphate buffer with increasing amount of liposomes

| | $\mu_{eff} \cdot 10^8$, $m^2 V^{-1} s^{-1}$ | | | | USP tailing (at 5% height) | | | |
|---|---|---|---|---|---|---|---|---|
| % lip | Ambroxol | Maraviroc | Canagliflozin | Deferasirox | Ambroxol | Maraviroc | Canagliflozin | Deferasirox |
| 0 | 1.60 ± 0.01 | 1.00 ± 0.01 | - | -1.71 ± 0.01 | 3.10 | 3.63 | - | 0.55 |
| 2 | 1.53 ± 0.01 | 0.99 ± 0.02 | -0.75 ± 0.01 | -1.71 ± 0.00 | 11.6 | 4.79 | 1.98 | 0.55 |
| 4 | 1.37 ± 0.02 | 0.97 ± 0.01 | -1.38 ± 0.02 | -1.72 ± 0.00 | 12.6 | 4.88 | 1.92 | 0.55 |
| 6 | 1.14 ± 0.03 | 0.95 ± 0.02 | -1.88 ± 0.02 | -1.73 ± 0.00 | ND* | 5.45 | 1.87 | 0.56 |
| 8 | - | 0.95 ± 0.01 | -2.26 ± 0.03 | -1.74 ± 0.00 | - | 6.42 | 2.00 | 0.57 |
| 10 | - | 0.90 ± 0.02 | -2.58 ± 0.03 | -1.75 ± 0.00 | - | 8.00 | 1.93 | 0.59 |

ND* – not determined due to a partial overlap with another peak

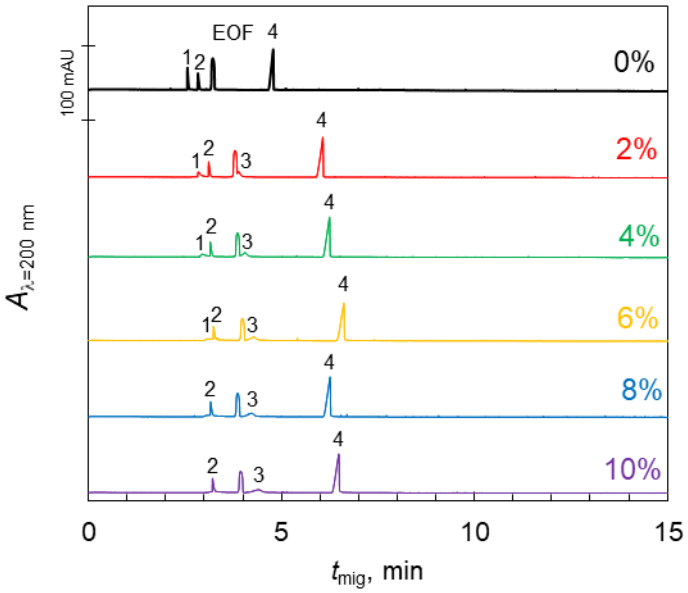


**Fig. 1** – Preliminary experiment of API-liposome interactions;
BGE: 10 mM sodium phosphate buffer at pH 7.10 with increasing amount of DPPC:DPPG (3:1) liposomes; API mixture contains ambroxol hydrochloride (1), maraviroc (2), canagliflozin (3) and deferasirox (4) at 0.1 mg/ml concentration

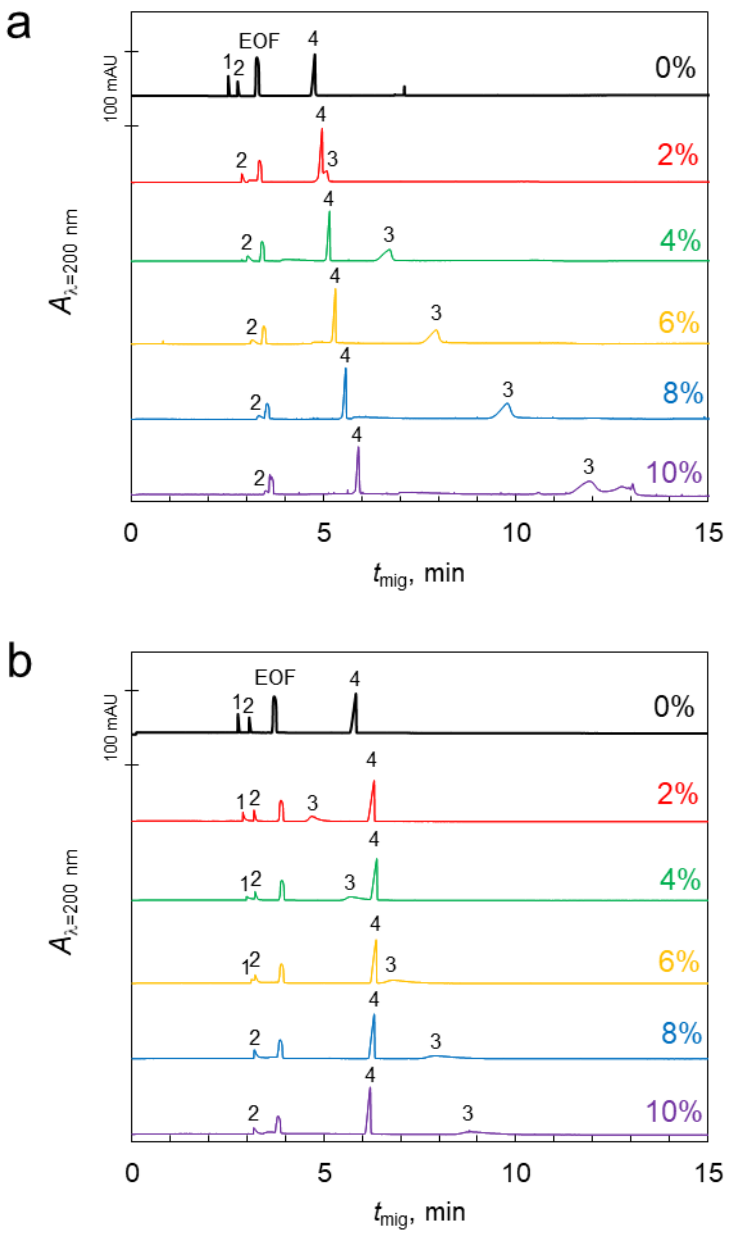


**Fig. 2** – Experiment of API-liposome interactions;
BGE: 10 mM sodium phosphate buffer at pH 7.10 with increasing amount of liposomes from bovine liver **(a)** or bovine heart **(b)** extracts; API mixture contains ambroxol hydrochloride (1), maraviroc (2), canagliflozin (3) and deferasirox (4) at 0.1 mg/ml concentration

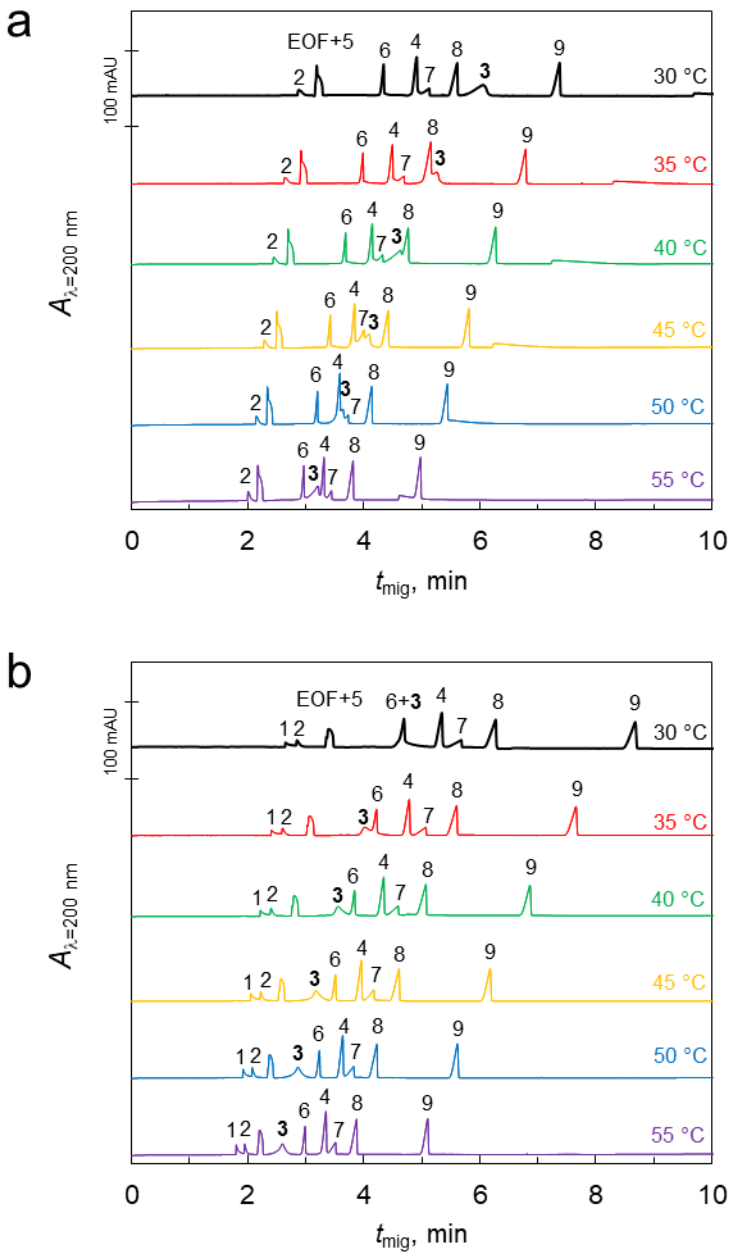


**Fig. 3** – Experiment of API-liposome interactions with increasing temperature during measurements; BGE: 10 mM sodium phosphate buffer at pH 7.10 with 4 % of liposomes from bovine liver **(a)** or bovine heart **(b)** extracts; API mixture contains ambroxol hydrochloride (1), maraviroc (2), canagliflozin (3), deferasirox (4), aprepitant (5), atorvastatin calcium (6), febuxostat (7), ibuprofen (8) and valsartan (9) at 0.1 mg/ml concentration

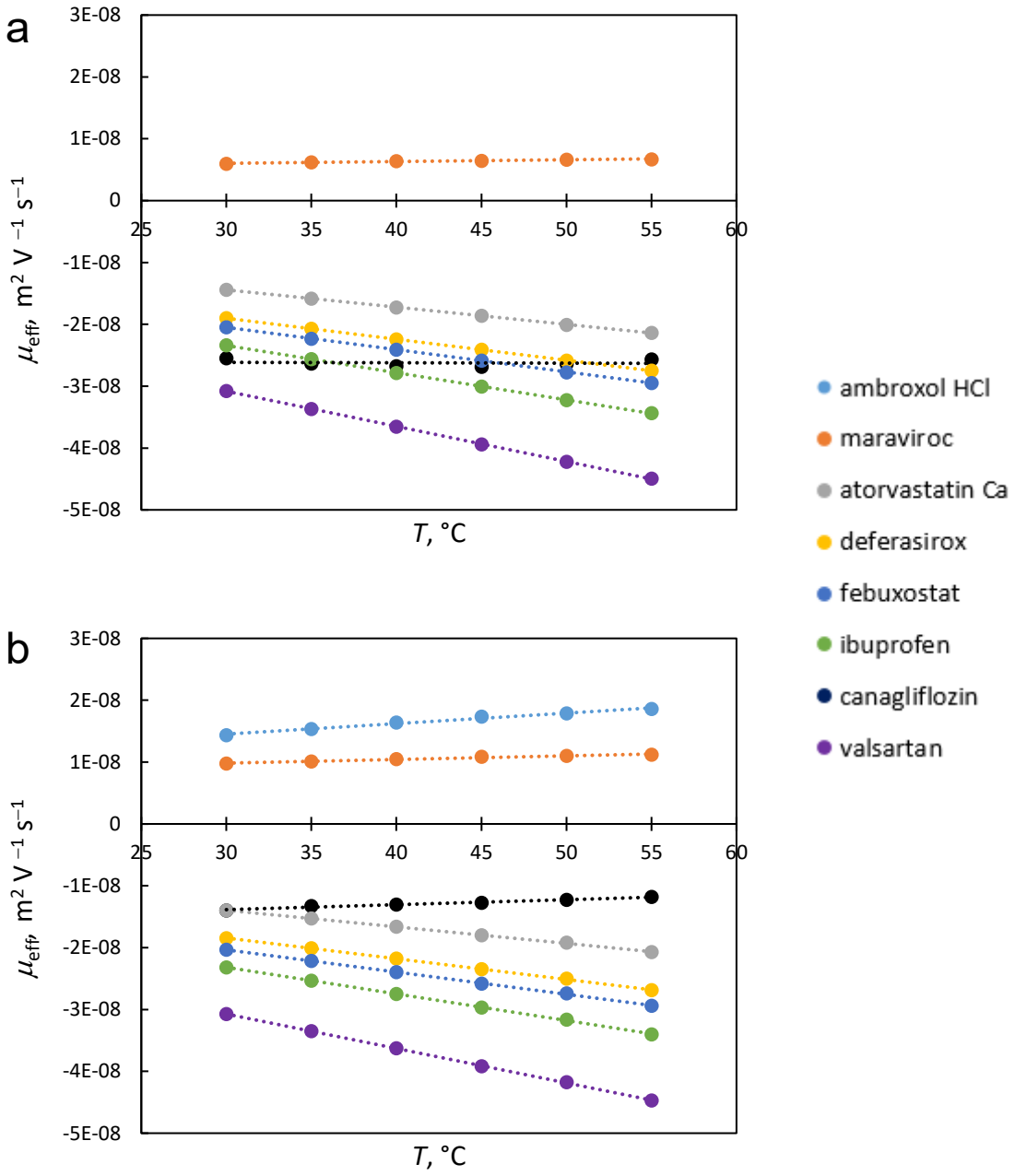


**Fig. 4** – Effective mobilities of individual APIs with increasing temperature during measurements; BGE: 10 mM sodium phosphate buffer at pH 7.10 with 4 % of liposomes from bovine liver **(a)** or bovine heart **(b)** extracts; API mixture contains ambroxol hydrochloride, maraviroc, canagliflozin, deferasirox, aprepitant, atorvastatin calcium, febuxostat, ibuprofen and valsartan at 0.1 mg/ml concentration

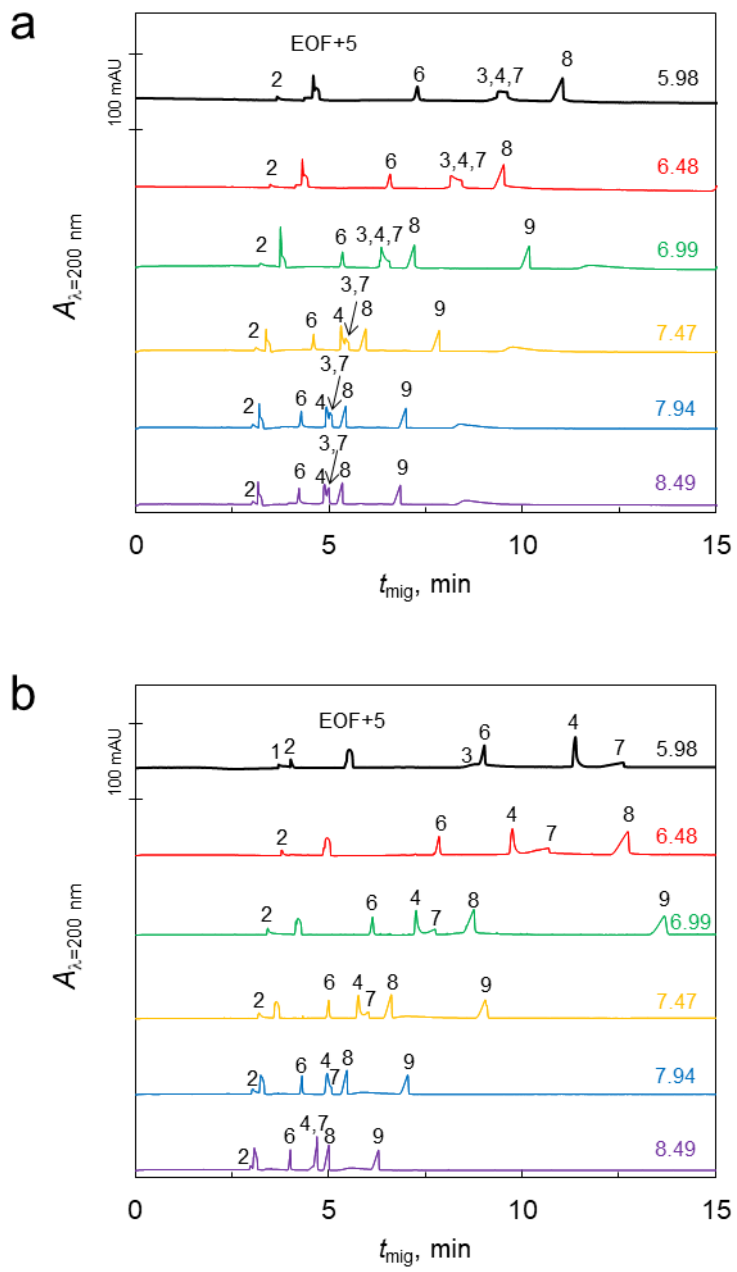


**Fig. 5** – Experiment of API-liposome interactions with increasing pH of the BGE;
BGE: sodium phosphate buffer at constant ionic strength (I = 20 mM) with 4 % of liposomes from bovine liver **(a)** or bovine heart **(b)** extracts; API mixture contains ambroxol hydrochloride (1), maraviroc (2), canagliflozin (3), deferasirox (4), aprepitant (5), atorvastatin calcium (6), febuxostat (7), ibuprofen (8) and valsartan (9) at 0.1 mg/ml concentration