# Università degli Studi di Roma "La Sapienza"

## Facoltà di Scienze Matematiche Fisiche e Naturali
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Tesi di Dottorato in Scienze Chimiche
(XVIII Ciclo)

# A Capillary Electrophoretic Approach for Studying the Interactions Between Biomolecules and Molecular Aggregates


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# Chapter 1- *Molecular Aggregates*

## *1.1 Introduction*

In recent years molecular aggregates such as micelles and liposomes have played a fundamental role in rapidly emerging achievements at the interface between chemistry, physics and biology.[1] The peculiar functions expressed by molecular aggregates are due to the organization of several monomers into a new system capable of functions, absent in the monomers. In this respect these systems mimic biological membranes where the interplay between molecular self-organization and molecular recognition of the individual constituents leads to the construction of complex and perfect systems capable of many specific functions that are fundamental to the cell life;[2] however the organization and the capabilities of molecular aggregates besides offering excellent membrane models, found application in many different fields.

## *1.2 Amphyphiles and Molecular Aggregates*

Molecular aggregates, like micelles and vesicles, are aggregates that arise from the assembly of amphiphilic molecules.

Molecules can be roughly divided into polar and non-polar ones according to the symmetry and distribution of their electronic clouds. Polar molecules are soluble in polar solvent and insoluble in non-polar and vice versa. Some molecules, however, possess at the same time a polar and a non-polar residues; these molecules are called amphiphiles and owing to the lyophobic and lyophilic interactions they can self-assemble and form organised structures in specific solvents in which they are dispersed. In these self-assembled structures, molecules are oriented in such a way that the polar portion of the molecule is in contact with the polar environment and shield the non-polar part, and vice versa (Figure 1).

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[1] (a) Lehn, J.M. *Supramolecular Chemistry, concepts and perspectives;* Verlag Chemie: Weinheim**, 1995**. (b) Lehn, J.M. *Science* **1985**, *227*, 849-856; (c) Lehn, J.M. *Angew. Chem. Int. Ed. Engl*. **1988**, *27*, 89-112.

[2] Alberts, B.; Bray, D.; Lewis, J.; Raft, M.; Roberts, K.; Watson, J.D. *Molecular Biology of the Cell*; Garland: New York, **1983**.

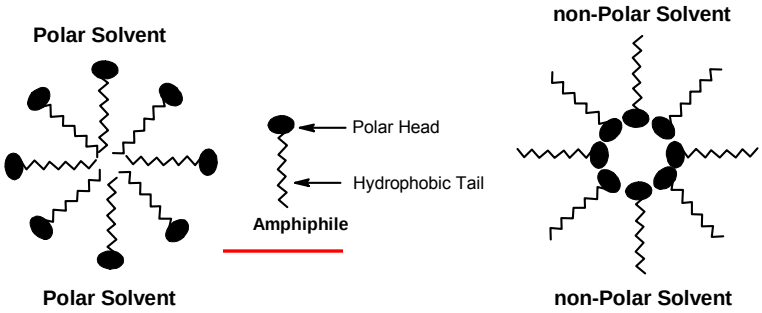


**Figure 1:** Schematic representation of lyophobic and lyophilic interactions. Amphiphiles expose either the polar head or the hydrophobic tail to polar and non-polar solvent, respectively, in order to minimize the lyophobic and the lyophilic interactions.

These aggregates are generally dispersed in water so we speak of hydrophobic and hydrophilic interactions. Depending on the features of the amphiphile and on the parameters such as temperature, ionic strength and concentration of surfactant, the aggregates formed in solution can be very different; among the possible kinds of aggregates there are micelles, planar bilayer, vesicles (or liposomes) and liquid crystals. Because this thesis takes into consideration micelles and liposomes, a short description of these aggregates is given below.

### *1.3 Micelles*

Amphiphiles spontaneously form micelles in solution above a certain concentration of surfactant, the critical micelle concentration (*cmc*), one of the characterizing parameters of amphiphiles.[3]

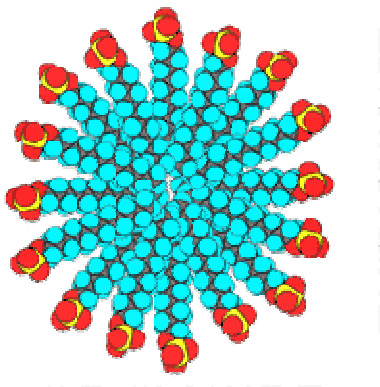

**Figure 2:** Schematic representation of a micelle, with the hydrocarbon tails pointing inside the aggregate and the polar head groups exposed to the polar solvent.

[3] Fuhrhop, J.H.; Koning, S. *Membranes and Molecular Assemblies: the Synkinetic Approach*, Editor: Stoddart, J.F., FRS, The Royal Society of Chemistry, **1994**.

Micellar aggregates present a polar external surface (consisting of the polar heads of the amphiphiles) exposed to water and a hydrophobic core (formed by the hydrophobic tails of the amphiphiles) as described in Figure 2. According to the molecular structure of the amphiphile and the experimental conditions (temperature, pH, ionic strength, concentration), micelles may have different shapes (spherical, cylindrical, ellipsoidal) and dimensions (hydrodynamic radius between 10 and 40 nm). Micelles are dynamic structures in which the monomer exchanges rapidly between the aggregate and the bulk phase ($10^{-6}$ s);[4] moreover inside the micelle the monomer has appreciable mobility, similar to those characterizing liquid paraffins.[5]

The *Krafft point*[6] is a parameter that individuates the conditions of temperature and concentration above which it is possible to have micelles in solution. This is defined as the temperature at which a rapidly increase of solubility of a surfactant in water begins to appear due to micelle formation.

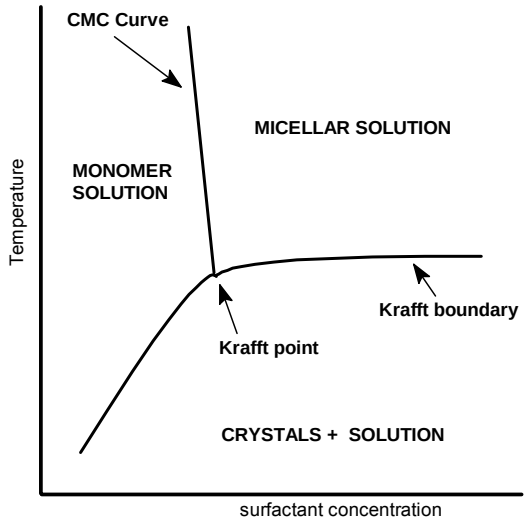


**Figure 3:** Phase diagram of the system surfactant/water. The Krafft point is the point where the three curves meet.

In the phase diagram of the surfactant/solvent system the Krafft point is regarded as the triple point at which a ternary equilibrium is established among crystals, monomeric surfactant and micellized surfactant. In the phase diagram reported in Figure 3 it can be

[4] Frindi, M.; Michels, B.; Zana, R. *J. Phys. Chem*. **1992**, *96*, 8137-8141.
[5] Williams, E.; Sears, B.; Allerhand, A.; Cordes, E.H. *J. Am. Chem. Soc*. **1973**, *95*, 4871-4873.
[6] *Surfactant Science Series-Cationic Surfactants, Physical Chemistry*, Vol. 37, ed. by D.N. Rubingh and P.M. Holland, Marcel Dekker, New York.

seen that: a) the curve separating the zone relative to the surfactant in the monomeric form from that relative to the surfactant in micellar form represents the variation of *cmc* as a function of temperature; b) the curve separating the surfactant in the monomeric form from the crystals represents the solubility of the surfactant below the Krafft point; c) the curve separating the surfactant in the micellar from the crystals represents the solubility of the surfactant above the Krafft point and it is also called the *Krafft boundary*. These three equilibrium boundaries meet at the Krafft point. Moreover, along the Krafft boundary the temperature at which a surfactant is completely soluble at a certain concentration is called the *Krafft temperature* for that concentration of surfactant.

Another parameter that characterizes micelles is the mean number of monomers that give raise to the aggregate, the mean aggregation number, *N*. This parameter depends on the nature and concentration of the amphiphile and on other factors such as temperature and ionic strength of the medium.

Micelles are a thermodynamically stable state and take place in solution by simply dissolving the amphiphiles in water, they usually do not change with time and they are broken by dilution.

### *1.4 Liposomes*

Liposomes or vesicles are self assembled colloidal particles in which bilayered membranes composed from natural and synthetic amphiphilic molecules encapsulate a fraction of the aqueous solution in which they are dispersed[7] as schematically shown in Figure 4.

[7] Lasic, D.; Templeton, N.S. *Advanced Drug Deliv. Rev*. **1996,** *20,* 221-226.

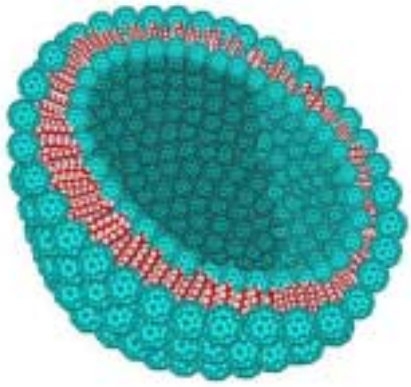

**Figure 4.** Representation of a section of a liposome. It can be noticed the lipidic double layer and the internal cavity.

Liposomes are not in a thermodynamically stable state so they cannot form spontaneously;[8] they are formed only if some energy (sonication, extrusion or homogenisation) is supplied to the system. After liposomes are formed they are in general not colloidally stable and slowly aggregate and fuse into larger aggregates characterized by a lower curvature and by a larger number of double layers. Because liposomes are in a kinetically and not in a thermodynamically stable state, they do not break upon dilution. A characteristic of the vesicles is the *main transition temperature* ($T_m$).[9] The main transition temperature is a parameter characterising the fluidity of the membrane concerning the transition from the fluid phase ($L_\alpha$) to the gel phase ($P_\beta$).

Liposomes obtained by the aggregation of amphiphiles in water, can have different dimensions and number of double layers. Liposomes can be mainly classified into three groups depending on size and morphology:[10] 1) Small Unilamellar Vesicles (SUV; below 50-80 nm of hydrodynamic diameter); 2) Large Unilamellar Vesicles (LUV; 80-1000 nm); 3) Multilamellar Vesicles (MLV) (Figure 5).

[8] Luisi, P.L. *J.Chem.Educ.* **2001**, *78*, 380-384.
[9] Sackmann, E., *Physical Basis of Self Organization and Function of Membrane: Physics of Vesicles,* in: Handbook of Biogical Physics chapter 5, Vol. 1 ed. by R. Lipowsky and E. Sackmann, **1995**, Elsevier Science B.V.
[10] *Liposomes*: *A Practical Approach,* in D. Rickwood and B.D. Hames (eds.); Practical Approach Series, Oil Press at Oxford University Press: Oxford, **1990**.

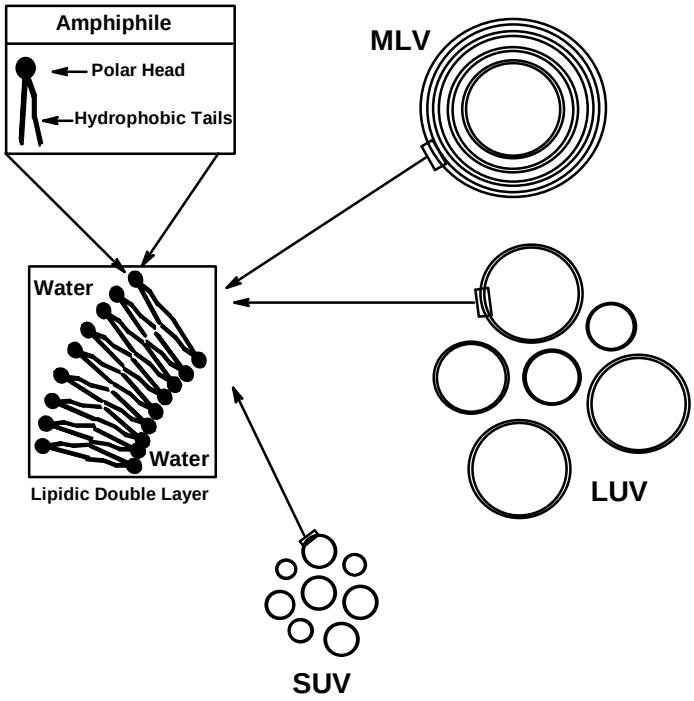


**Figure 5.** Schematic representation of liposomes. With respect to the size, small (below 50-80 nm, depending on lipid composition) and large vesicles (from 80-1000 nm and giant-size from 1 to 50 μm) can be distinguished, while with respect to lamellarity, unilamellar and multilamellar liposomes are defined.

Unilamellar Liposomes are well used as models of biological membranes and can be produced by many different techniques such as sonication[11] of the MLV dispersion or extrusion in which the MLV dispersion is passed under pressure (150-700 psi) through a polycarbonate filter of appropriate pore size.[12] The extrusion technique in combination with the freeze-thaw procedure is especially advantageous and fast to obtain a homogeneous monodispersion of unilamellar vesicles[13] with a diameter in the range of 50-400 nm; injection of a solution of the lipids dissolved in ethanol[14] or in ether[15] in water or the solubilization of lipids by surfactants are useful to obtain unilamellar vesicles.[16] Once liposomes of appropriate dimensions are prepared it is possible to determine some parameters characterizing the aggregates. Size of liposomes is

[11] Huang, C.H. *Biochemistry* **1969**, *8*, 344-352.
[12] (a) Barenholz, Y.; Anselem, S.; Lichtenberg D. *FEBS Lett.* **1979**, *99*, 210-214. (b) Hope, M.J.; Bally, M.B.; Webb, G.; Cullis, P.R. *Biochim. Biophys. Acta* **1985**, *812*, 55-65.
[13] Hope, M.J.; Mayer, L.D.; Cullis, P.R. *Biochim. Biophys. Acta* **1986**, *858*, 161-168.
[14] Kremer, J.M.H.; Esker, M.W.J.; Pathmamanohonovan, C.; Weissmann, G. *Biochemistry* **1977,** *16*, 3932-3935.
[15] Deamer, D.W. in *Liposome Technology,* G. Gregoriadis Ed.; CRC Press: Boca Raton Florida, **1984**; Vol. 1, p. 29.
[16] Mimms, L.T.; Zampighi, G.; Nozaki, Y.; Tanford, C.; Reynolds, J.A. *Biochemistry* **1981**, *20*, 833-840.

characterized by Dynamic Light Scattering (DLS)[17] and Freeze-Fracture Electron Microscopy,[18] besides the volume entrapped in the internal cavity of the liposomes can be determined.[19] Elucidation of the bilayer structure and the stability can be obtained by using small-angle X-ray scattering (SAXS).[20] Differential scanning calorimetry[21] and fluorescence[22] have been used to characterize the $T_m$ of the liposomes. Surface charge can be characterized by measuring zeta-potential, or using adsorptivity measurements and magnetic resonance methods.[23]

Capillary Electrophoresis is a recent approach for studying liposomal properties such as membrane fluidity and rigidity, size distributions, phospholipid distribution in the membrane, membrane disruption, and surface charge density.[24] The applied voltages used are well below that required to electroporate a membrane,[25] thus this technique can be used to qualitatively and quantitatively characterize lipid vesicle dispersions. The migration time of the vesicles can infer general information about the net surface charge while peak width give information about the polydispersity of the liposome preparation.

### *1.5 Prediction of the Morphology of the Aggregates: a Theoretical Approach*

A major goal in amphiphiles chemistry is the prediction of the aggregate type on the basis of their molecular structures.

Thus a dimensionless critical packing parameter P (equation 1) has been defined by Israelachvili[26] who correlated the geometric features of the amphiphile to the structure of its aggregates. The packing parameter P has a magnitude which depends on the

[17] Maulucci, G.; De Spirito, M.; Arcovito, G.; Boffi, F.; Castellano Congiu, A.; Briganti, G. *Biophys. J.* **2005**, *88*, 3545-3550.
[18] van Venetie, R.; Leunissen-Bijvelt, J.; Verkleij, A.J.; Ververgaert, P.H.J.T. *J. Microsc.* **1980**, *118*, 401-408.
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[20] Muller, M.; Mackeben, S.; Muller-Goymann, C.C. *Int. J. Pharm*. **2004**, *274*, 139-148.
[21] Ryhanen, S.J.; Pakkanen, A.L.; Saily, J.; Bello, C.; Mancini, G.; Kinnunen, P.*J. Phys. Chem. B* **2002**, *106*, 11694-11697.
[22] Gliozzi, A.; Relini, A.; Chong, P.L.G. *J. Membr. Sci*. **2002**, *206*, 131-147.
[23] Venkatesan, N.; Vyas, S.P. *Int. J Pharm*. **2000**, *203*, 169-177.
[24] (a) Kawakami, K.; Nishihara, Y.; Hirano, K. *Langmuir*, **1999**, *15*, 1893-1895. (b) Roberts, M.A.; Lo Cascio-Brown, L.; MacCrehan, W.A.; Durst, R.A. Anal. Chem. **1996**, *68*, 3434-3440. (c) Radko, S.P.; Stastna M.; Chrambach, A. *Anal Chem*. **2000**, *72*, 5955-5960. (d) Radko, S.; Stastna, M.; Chrambac, A.*J. Chromatogr. B* **2001**, *761*, 69-75. (e) Wiedmer, S.K.; Hautala, J.; Holopainen, J.M.; Kinnunen, P.K.; Riekkola, M.L. *Electrophoresis* **2001**, *22*, 1305-1313. (f) Tsukagoshi, K.; Okumura, Y.; Nakajima, R. *J. Chromatogr. A*, **1998**, *813*, 402-407. (g) Duffy, C.F.; Gafoor, S.; Richardes, D.P.; Admadzadeh, H.; O'Kennedy, R.; Arriaga, E.A. *Anal Chem*. **2001**, *73*, 1855-1861.
[25] Karlsson, M.; Nolkrantz, K.; Davidson, M. J.; Stromberg, A.; Ryttsen, F.; Akerman, B.; Orwar, O. *Anal. Chem.* **2000**, *72*, 5857–5862.
[26] Israelachvili, J.N. *Intramolecular and Surfaces Forces*; Academic Press, **1991**.

volume ν of the fluid and incompressible hydrocarbon chains, the cross-sectional headgroup area ($a_o$) per amphiphile exposed to the solvent and the critical chain length ($l_c$ ~ maximum effective chain length):

$$P=\nu/a_o \cdot l_c \qquad \text{(eqn. 1)}$$

In good approximation, ν and $l_c$ for a saturated hydrocarbon chain of n carbon atoms are given by:

$$\nu \approx (27.4 + 26.9\ n)\text{Å}^3$$

$$l_c \approx l_{max} \approx (1.5 + 1.265\ n)\text{Å}^3$$

The morphology of the aggregates is related to the magnitude of P as summarized in Table 1 and Figure 6.

**Table 1.** Morphology of the aggregates correlated with the packing parameter.

| P | General Surfactant Type | Expected Organization |
|---|---|---|
| <0.33 | Amphiphiles with one aliphatic chain and a relatively large polar head group | Spherical or ellipsoidal micelles |
| 0.33-0.5 | Amphiphiles with a relatively small polar head group | Cylindrical micelles |
| 0.5-1 | Amphiphiles with a large polar head group and flexible chain | Vesicles |
| 1 | Amphiphiles with two aliphatic chains and a small polar head group | Planar bilayers |

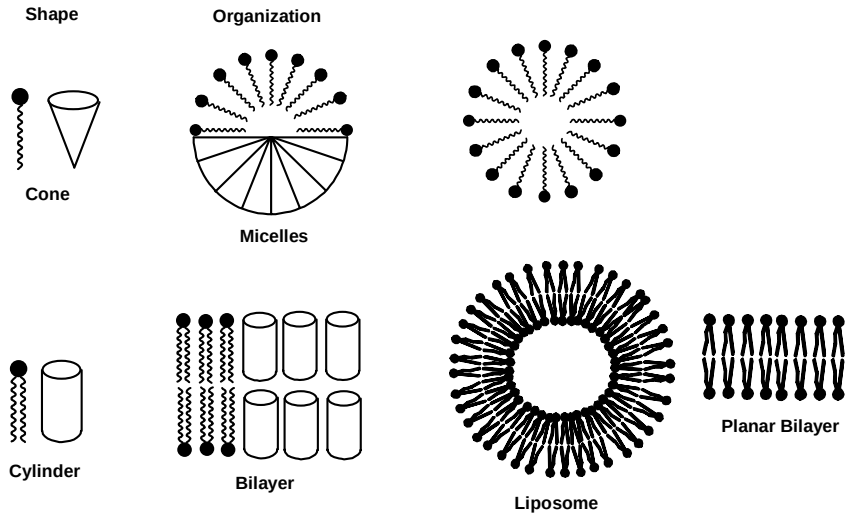


**Figure 6.** Self aggregation of amphiphiles: effect of the critical packing parameter P on the morphology of the aggregates. Depending on molecular shape of the amphiphile, the temperature and the condition in the lipid-water mixture (concentration and ionic strength), amphiphiles may self assemble into different colloidal particles.

The packing parameter can be quite useful in the prediction of the shape of the aggregate but has to be used carefully because it may fail in the case of complex amphiphiles. Even other factors, not taken into account by Israelachvili model, influence the aggregation process.

### *1.6 Applications of Molecular Aggregates*

Due to their structure, chemical composition, colloidal size and amphiphilic character, molecular aggregates such as micelles and liposomes, exhibit several properties which are useful in various applications.

Micelles have been largely employed as biological membrane models[27] and as carriers in the drug-delivery[28] for their capability of solubilizing hydrophobic drugs thus increasing bioavailability and long-circulating.[29] Micellar catalysis of some organic reactions has provided models for enzyme behaviour.[30]

Liposomes are a very useful model, reagent and tool in various scientific disciplines, including biophysics (properties of cell membranes), chemistry (catalysis, energy conversion, photosynthesis), colloid science (stability and thermodynamics of finite systems), biochemistry (recognition processes, function of membrane proteins) and biology (cell function, signal transduction, gene delivery).[31] The applied side includes liposomes as drug delivery vehicles in medicine,[32] adjuvants in vaccination,[33] signal enhancers/carriers in medical diagnostic[34] and analytical biochemistry,[35] solubilizers for various ingredients as well as penetration enhancers in cosmetic.[36] Liposome encapsulation in anticancer therapy can alter the spatial and temporal distribution of the encapsulated drug molecules in the body and may significantly reduce unwanted toxic

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[27] (a) Fresta, M.; Guccione, S.; Beccari, A.R.; Furieri, P.M.; Pugliesi, G. *Bioorg. Med. Chem*. **2002**, *10*, 3871-3889.
[28] Chakraborty, H.; Banerjee, R.; Sarker, M. *Biophys. Chem*. **2003**, *104*, 315-325.
[29] Torchilin, V.P.*J. Contr. Rel*. **2001**, *73*, 137-172.
[30] Price, S.E.; Jappar, D.; Lorenzo, P.; Saavedra, J.E.; Hrabie, J.A.; Davies, K.M. *Langmuir*, **2003**, *19*, 2096-2102.
[31] (a) Lasic, D.D. *Liposomes: From Physics to Application*, **1993**, Elsevier. (b) Lasic, D.D. *Am Sci*. **1992**, *80*, 20-31.
[32] Lasic, D.D. *TiBTech*, **1998**, *16*, 307-321.
[33] Gluck, R.; Mischler, R.; Finkel, B.; Que, J.U.; Scarpa, B.; Cryz, S.J. *Lancet*, **1994**, *344*, 160-163.
[34] Williams, B.D.; Sullivan, M.M.; Williams, K.E.; Williams, J.R.; Morgan, J.R. *Br. Med. J*. **1986**, *293*, 1144-1145.
[35] (a) Rongen, H.A.H.; Bult, A.; van Bennekom, W.P. *J. Immunol. Meth*. **1997**, *204*, 105-133. (b) McNamara, K.P.; Rosenzweig, Z. *Anal. Chem*. **1998**, *70*, 4853-4859.
[36] Barenholz, Y.; Lasic, D.D. *Handbook of Nonmedical Application of Liposomes*, **1996**, CRC, Boca Raton, FL.

side effects and increase the efficacy of the treatment.[37] In gene therapy cationic liposomes have been shown to complex (negatively charged) DNA, and such complexes were able to transfect cells *in vitro*,[38] resulting in the expression of the protein encoded in the DNA plasmid in the target cells. Liposomes have been also employed in food industry[39] as sustained release systems in the fermentative processes and in the ecological field[40] as solvent agent for the oil stain in the sea-water; liposomes containing membrane anchored chelators could be used to remove toxic or radioactive metal ions from solutions.

Recently liposomes have been found applications in analytical chemistry; they have been used as coating material in liquid chromatography[41] and as carriers and coating material in capillary electrophoresis.[42] Some applications of vesicles in CE are not aimed at better separation of the analytes, but rather at investigating the chemical/physical affinity of bioactive compounds for membranes.

Moreover CE is a powerful technique to characterize liposome formulations, for both quality monitoring and direct quantitation of released encapsulated materials; in fact liposomes are currently applied as drug delivery vehicles and one of the important requirements for these formulations is good stability without significant aggregation or loss of entrapped drug.

### *1.7 Aim of this Work*

Since the interactions between biomolecules and cell membrane play a key role in many cellular processes[43] the interaction between peptides and proteins and lipid bilayers has been a growing area of interest in the last years.[44] Interactions of water soluble proteins

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[37] Gabizon A. *Liposomes as a drug delivery system in cancer therapy,* in Drug Carrier System eds. F. D. H. Roerdink and A.M. Kron: Wiley, Chichester, **1989**; 185-211.

[38] van der Veen, A.H.; ten Hagen, T.L.M.; van Tiel, S.; Seynhaeve, A.L.B.; Eggermont, A.M.M. in *Progress in Drug Delivery Systems* **1996**, Vol. 5 Hirota, S. ed., pp. 101–104, Biomedical Research Foundation, Tokyo, Japan

[39] Keller, B.C. *Trend Food Sci. Tech*. **2001**, *12*, 25-31.

[40] (a) Gatt, S.; Bercovier, J.H.; Barenholz, Y. *On Site Bioreclamation* eds. R.E. Hinchee and R.F. Olfensbuttal: Butterworth, Stoneham, **1991**; 293-312. (b) Dutton, G. *Gen. Eng. News* **1993**, *13*, 6-9.

[41] Gomez-Hens, A.; Fernandez-Romero, J.M. *TiAChem*, **2005**, *43*, 9-19.

[42] (a) Wiedmer, S.K; Jussila, M.S.; Riekkola, M.L. *TiAChem*, **2004**, *23*, 562-582. (b) Owen, R.L.; Strasters, J.K.; Breyer, E.D. *Electrophoresis*, **2005**, *26*, 735-751.

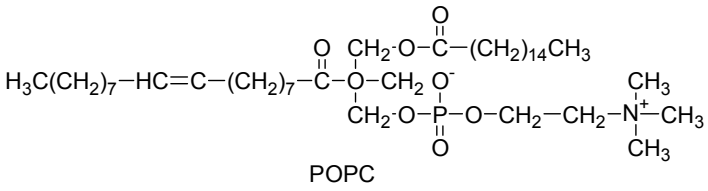
[43] (a) Heldin, C.H; Purton, M. *Signal transduction* **1996**, Chapman and Hall, London. (b) Hancock, J.T. *Cell Signaling*, **1997**, Addison Wesley, Longman, Essex.

[44] (a) White, S.H.; Wimley, W.C.; Ladokhin, A.S.; Hristova, K. *Meth. Enzymol*. **1998**, *295*, 62-87. (b) Epand, R.M.; Vogel, H.J. *Biochim. Biophys. Acta* **1999**, *1462*, 11-28.

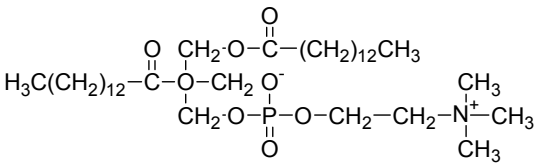
with membranes are deeply involved in many biological processes including energy and signal transduction, regulation of biological activity and transport processes.[45] The use of several carefully chosen synthetic or naturally occurring peptide segments from integral membrane proteins or from membrane binding proteins has helped to clarify the principles that govern molecular recognition between proteins and membrane.[46]

Aim of this thesis is to investigate the potentiality of capillary electrophoresis for studying peptide/protein-membrane models interactions. With respect to the other static technique, the dynamically flowing environment offered by capillary electrophoresis on the interactions of lipid vesicles with biologically active molecules, such as drugs, proteins or peptides, can be very indicative of transport through membranes and can provide insight into the physiological in vivo behaviour of these bioactive compounds. At this aim we investigated by different strategies the interactions between peptides/proteins and liposomes.

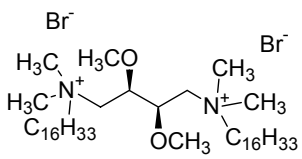
We used liposomes formed by a natural phospholipid 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) and mixed liposomes formed by POPC or 1,2-dimiristoyl-sn-glycero-3-phosphocholine (DMPC) and the cationic gemini surfactant (2S,3S)-2,3-dimethoxy-1,4-bis(*N*,*N*-dimethyl-*N*-hexadecylammonium) butane dibromide (SS), and studied, through their separation capabilities, the interactions with three closely related peptides, namely angiotensin I, angiotensin II and angiotensin III and some basic proteins, lysozyme, cytochrome C, ribonuclease A and α-chymotrypsinogen A.



POPC



DMPC



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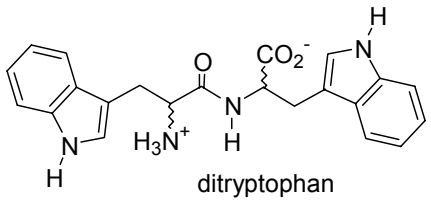
[45] Jutila, A.; Rytomaa, M.; Kinnunen, P.K.J. Chem. Phys. Lip. **1994**, *73*, 181-207.
[46] (a) Deber, C.M.; Liu, L.P.; Wang, C.*J. Pept. Res*. **1999**, *54*, 200-205. (b) Shai, Y.*J. Membr. Biol*. **2001**, *182*, 91-104. (c) Wimley, W.C.; White, S.H. *Nat. Struct. Biol*. **1996**, *3*, 842-848.

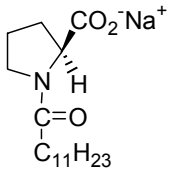
Since the three closely related peptides display similar electrophoretic behaviour, differences in their migration times in the presence of liposome dispersions could allow us to obtain information about the kind of interactions established between peptides and liposomes.

Concerning the four basic proteins it can be noted that, in the absence of liposome dispersion, they are irreversibly absorbed onto the capillary wall. Thus, the observation of discrete peaks in the presence of liposome dispersion could be indicative of the protein-liposome interactions.

In the second part of the thesis work, we took into consideration chiral recognition as a specific aspect of the biomembrane/peptide interactions and investigated as model systems the four diastereomers of ditryptophan associated with the aggregates formed by a chiral amidic surfactant derived from proline, namely sodium *N*-dodecanoyl-L-prolinate (SDP).




ditryptophan




sodium N-dodecanoyl-L-prolinate

Aromatic peptides have been chosen because the high affinity of aromatic amino acids for lipidic aggregates. In particular, tryptophan is known to play a role in the interaction of membrane proteins with the lipidic double layer.[47] Moreover the presence of aromatic portions in the peptide allows the detection by the UV-vis detector of CE.

Sodium *N*-dodecanoyl-L-prolinate is not a natural amphiphile and it was chosen in this study because it is characterized by an high extent of organization and the interactions involved in its micellar aggregates are similar to those responsible for the aggregation and organization of the aggregates formed by natural lipids.[48]

At the same time this work has not underestimated the suitability of capillary electrophoresis at separating peptide and proteins using molecular aggregates in bare-fused silica capillaries. The growth of interest of life science such as proteomics which

[47] Yau, W.M.; Wimley, W.C.; Gawrish, K.; White, S.H. *Biochemistry* **1998**, *37*, 14713-14718.
[48] Borocci, S.; Mancini, G.; Cerichelli, G.; Luchetti, L. *Langmuir* **1999**, *15*, 2627-2630.

refers to the expression of proteins by cells and the consequent purity requirements of both natural and biotechnologies proteins, requires the development of advanced chromatographic and electrophoretic techniques for the separation and identification of the expressed proteins. CE is a versatile and powerful technique that could offer rapid analysis time and efficient separation of proteins but the molecular complexity of peptides and proteins and the multifunctional character of amino acids require particular attention in selecting the capillary tube and the composition of the electrolyte solution employed for the separation of the proteins and peptides by capillary electrophoresis.

# Chapter 2-*Capillary Electrophoresis and Molecular Aggregates*

## *2.1 Capillary Electrophoresis*

The rapid analysis time, efficient separation, low solvent and sample volume required by capillary electrophoresis, are responsible for the growing interest of this relative new technique in bioanalytical and pharmaceutical applications. In the present chapter the basic instrumental aspects, separation principles and recent applications of capillary electrophoresis will be summarized.

Capillary electrophoresis (CE) is a technique based on the electro-migration of charged and neutral species under the influence of an electric field generated into a capillary tube. The instrumentation consists of a capillary tube of internal diameter varying from 25 to 100 μm, whose extremities are immersed into an electrolyte solution (BGE), an high voltage power supply (usually up to 30 kV) and a detector (UV-vis, fluorescence or mass spectrometer) for the online detection of the species (Figure 1).

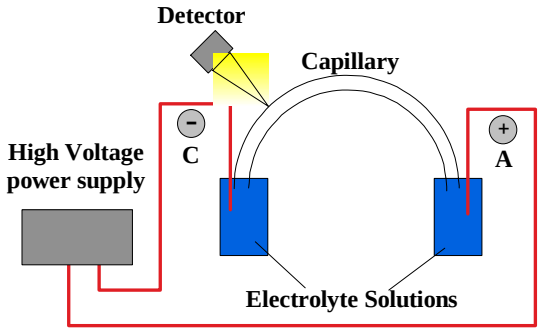



**Figure 1.** Scheme of capillary electrophoresis system. A=anode and C=cathode.

In an electric field, positively charged species tend to migrate toward the negative electrode (cathode) and negatively charged species tend to migrate toward the positive electrode (anode) with an electrophoretic mobility depending on their charge/size ratio. However, in the presence of the electro-osmotic flow (EOF) all species generally migrate toward the cathode, positively charged species adding their electrophoretic mobility to that of electro-osmotic flow and negative ones subtrahend their electrophoretic mobility to that of electro-osmotic flow. All neutral species migrate toward the cathode with the same electrophoretic mobility of the EOF (Figure 2).

The effective mobility of the species ($\mu_{eff}$) is:

$$\mu_{eff}=\mu_{eof}+\mu_{app} \qquad \text{(eqn. 1)}$$

where $\mu_{eof}$ is the mobility of the electro-osmotic flow and $\mu_{app}$ is the apparent mobility of the species. The apparent electrophoretic mobility, $\mu_{app}$ can be easily calculated using the Helmoltz-von Smoluchowski equation in the following form:[1]

$$\mu_{app} = \frac{L_T \cdot L_D}{V \cdot t} \qquad (m^2/V.s) \qquad \text{(eqn. 2)}$$

where $L_T$ (in meters) is the total length of the capillary, $L_D$ (in meters) is the effective length of the capillary (distance from the end of the capillary where the sample are introduced to the detection windows), V is the applied voltage (in kilovolts) and t (in seconds) is the migration time of the analytes.

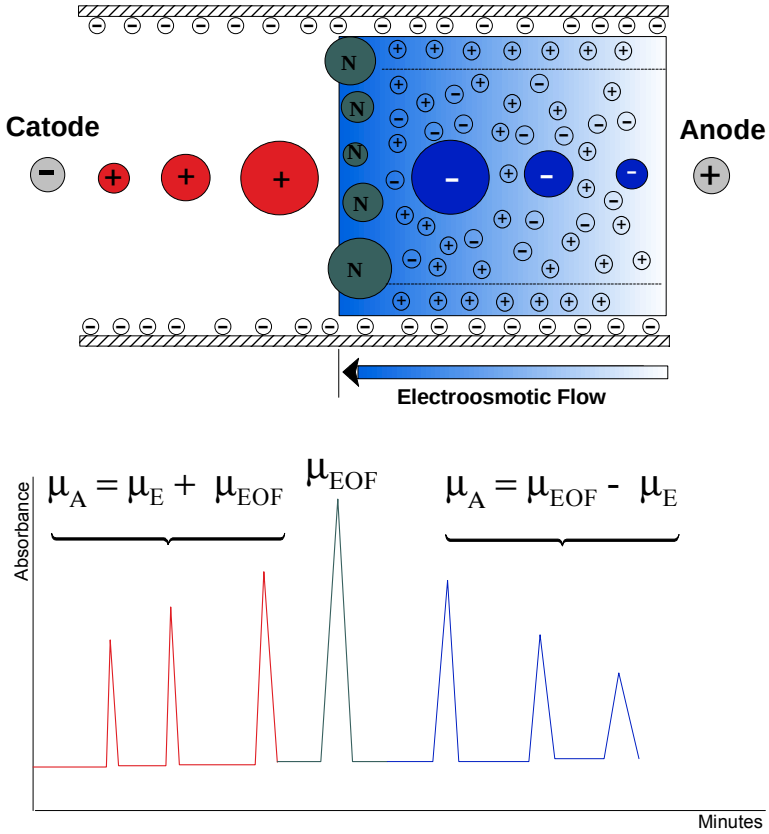



**Figure 2.** Schematic representation of the migration of the analytes into the capillary. Under the influence of an electric field and of the electro-osmotic flow, all the species (charged and neutral) migrate toward the cathode with their own electrophoretic mobility depending on the charge/size ratio.

[1] Corradini, D. *Encyclopaedia of Chromatography*, J. Cazes Ed., Marcel Dekker, Inc., New York, **2000**, 291-294.

The electro-osmotic flow is generated by the effect of the applied electric field across the tube on the uneven distribution of ions in the electric double layer at the interface between the capillary wall and the electrolyte solution. In bare fused silica capillaries, ionisable silanol groups are present at the surface of the capillary wall exposed to the electrolyte solution. In this case, the electric double layer is the result of the excess of cations in the solution in contact with the capillary tube to balance the negative charges on the wall arising from the ionisation of the silanol groups. Part of the excess cations are firmly held in the region of the double layer closer to the capillary wall (the compact or Stern layer; Figure 3) and are believed to be less hydrated than those in the diffusive region of the double layer.[2]

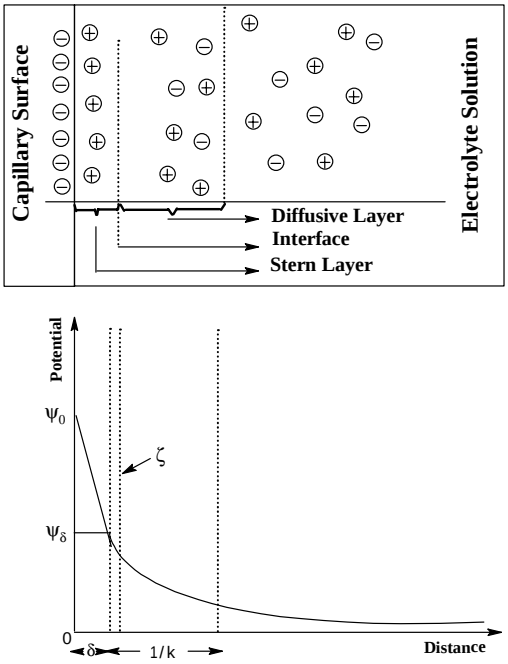



**Figure 3.** Schematic representation of the electric double layer at a solid-liquid interface and variation of potential with the distance from the solid surface: $\psi_o$, surface potential; $\psi_\delta$, potential at the Stern plane; $\xi$, potential at the plane of share (zeta potential); $\delta$, distance of the Stern plane from the surface (thickness of the Stern layer); $k^{-1}$, thickness of the diffuse region of the double layer.

When an electric field is applied across the capillary, the remaining excess cations in the diffusive part of the electric double layer move toward the cathode, dragging their hydration spheres with them. Because the molecules of water associated with the

[2] Heimenz, P.C. *Principle of Colloid and Surface Chemistry*, 2nd ed., Marcel Dekker, Inc., New York, **1986**, 677-735.

cations are in direct contact with the bulk solvent, all the electrolyte solution moves toward the cathode producing a pluglike flow having a flat velocity distribution across the capillary diameter.[3] The flow of liquid caused by the electro-osmosis displays a pluglike profile because the driving force is uniformly distributed along the capillary tube. Consequently, a uniform flow velocity vector occur across the capillary. The flow velocity approach zero only in the region of the double layer very close to the capillary surface.

The electro-osmotic mobility, $\mu_{eof}$ can be easily calculated by using equation 2:

$$\mu_{eof} = \frac{L_T \cdot L_D}{V \cdot t_{eof}} \qquad (m^2/V.s)$$

where $t_{eof}$ (in seconds) is the migration time of an electrically neutral marker substance. The electrically neutral marker substance employed to measure the mobility of the electro-osmotic flow has to fulfil the following requirements: the compound must be soluble in the electrolyte solution and neutral in a wide pH range and no interaction with the capillary wall or other sample components must occur. Some compounds that adequately serve as electrically neutral markers include benzyl alcohol, riboflavin, acetone, dimethyl formamide, dimethyl sulfoxide and mesityl oxide. Under constant composition of the electrolyte solution, the electro-osmotic flow depends on the magnitude of the zeta potential which is determined by many different factors, the most important being the ionisation of the silanol groups on the capillary wall, the charge density in the Stern layer and the thickness of the diffusive layer. Each of these factors depends on several variables, such as pH, specific absorption of ionic species in the compact region of the double layer, ionic strength, viscosity and temperature.

### *2.2 Molecular Aggregates in Capillary Electrophoresis: State of the Art*

Liposomes have found a variety of application in capillary electrophoresis. The first use of liposomes in capillary electrophoresis was reported by Hjerten and coworkers[4] for studying the interaction of liposomes with some models drugs and two octapeptides. Liposomes were employed as a pseudo-stationary phase that was introduced into a polyacrylamide coated electroosmosis-free capillary from the cathodic end to a zone

[3] Hjertén, S. *Chromatogr. Rev*. **1967**, *9*, 122-219.
[4] Zhang, Y.; Zhang, R.; Hjerten, S.; Lundhal, P. *Electrophoresis* **1995**, *16*, 1519-1523.

close to the detection windows; the decrease in the mobility of the analytes toward the anode, observed in the presence of the liposomes dispersion, was attributed to the interactions between negatively charged analytes and liposomes. Such approach, termed by Hjerten and co-workers liposome capillary electrophoresis (LCE), has been employed in a number of applications, including the determination of liposome-water partition coefficients for neutral aromatic solutes,[5] the study of interaction of a variety of neutral solutes with unilamellar liposomes composed by a mixture of two phospholipids,[6] the investigation of the interactions of a set of drugs with liposomes[7] and the evaluation of the binding constant with cationic liposomes of fluorescein conjugated 2-*O*-methylphosphorothionate antisense oligonucleotides.[8] Binding studies between drugs and liposomes have also been performed by frontal capillary electrophoresis[9] and by immobilized liposome affinity electrochromatography, using capillaries with immobilized liposomes at the inner wall of a bare fused-silica capillary.[10] Liposomes have also been employed as dynamic coating agents for bare fused-silica capillaries used in CE of proteins[11] and small molecules.[12] Moreover, LCE has been applied for separating uncharged solutes by a mechanism based on the distribution of analytes between an aqueous buffer phase and a liposome phase. Except in the work by Hjerten and co workers, such approach has been restricted to low molecular mass analytes such as benzene derivatives, steroids, phenols,[13] and riboflavine.[14]

Micelles have been largely employed in capillary electrophoresis for the separation of both charged and neutral molecules by a methodology based on the distribution of the analytes between the hydrophobic micelles formed by a surfactant and the electrolyte

[5] Burns, S.T.; Khaledi, M.G. *J. Pharm. Sci*. **2002**, *91*, 1601-1612.
[6] Burns, S.T.; Agbodjan, A.A.; Khaledi, M.G. *J. Chromatogr. A* **2002**, *973*, 167-196.
[7] Manetto, G.; Bellini, M.S.; Deyl, Z. *J. Chromatogr. A* **2003**, *990*, 205-214.
[8] McKeon, J.; Khaledi, M.G. *J. Chromatogr. A* **2003**, *1004*, 39-46.
[9] Kuroda, Y.; Watanabe, Y.; Shibukawa, A.; Nakagawa, T. *J. Pharm. Biomed. Anal*. **2003**, *30*, 1869-1877.
[10] Manetto, G.; Bellini, M.S.; Deyl, Z. *J. Chromatogr. A* **2003**, *990*, 281-289.
[11] Cunliffe, J.M.; Baryla, N.E.; Lucy, C.A. *Anal. Chem*. **2002**, *74*, 776-783.
[12] (a) Hautala, J.T.; Linden, M.V.; Wiedmer, S.K.; Ryhanen, S.J.; Saily, M.J.; Kinnunen, P.J.K.; Riekkola, M.L. *J. Chromatogr. A* **2003**, *1004*, 81-90. (b) Hautala, J.T.; Wiedmer, S.K.; Riekkola, M.L *Anal. Bioanal. Chem*. **2004**, *378*, 1769-1776.
[13] Wiedmer, S.K.; Hautala, J.T.; Holopainen, J.M.;Kinnunen, P.J.K.; Riekkola, M.L. *Electrophoresis* **2001**, *22*, 1305-1313.
[14] Roberts, M.A.; Lo Cascio-Brown, L.; MacCrehan, W.A.; Durst, R.A. Anal. Chem. **1996**, *68*, 3434-3440.

solution. The surfactant is incorporated into the electrolyte solution at concentration higher than its critical micelle concentration (cmc), so that micelle are formed. The methodology was introduced in 1984 by Terabe[15] and is termed either Micellar Electrokinetic Capillary Chromatography (MECC) or Micellar Electrokinetic Chromatography (MEKC).[16] When an anionic surfactant, such as sodium dodecyl sulphate (SDS), is employed the application of the electric field would force the micelles to migrate toward the anode but the electro-osmotic flow is larger than the electrophoretic migration of the micelle under neutral or basic conditions, therefore anionic SDS micelles migrate toward the cathode at retarded velocity. When a solute is injected into the capillary, it will be distributed between the micelles and the bulk solution. Therefore, the solute will migrate at a velocity depending on its distribution coefficient (K) into the micelle and on its electrophoretic mobility (only for a charged solute). The results is that all solutes migrate at a velocity ranged within two extreme values, i.e. the electro-osmotic mobility ($\mu_{eof}$; distribution coefficient $K = 0$) and the micelle mobility ($\mu_{mc}$; distribution coefficient $K = \infty$). The interactions between solute and micelles depend on several parameters, such as the micellar structure, temperature, pH and additives to aqueous phase. MEKC has been proven useful for the analysis of a variety of low-molecular weight species and it is largely employed for the separation of chiral solutes using chiral surfactants or stereoselective additives, such as modified ciclodextrins. Chiral surfactants mainly used in MEKC for chiral separation are as follows: amino acids derivatives, such as sodium *N*-dodecanoyl-L-valinate,[17] *N*-dodecanoyl-L-glutamate, digitonin [18] and bile salts.[19] On the other hand, MEKC exhibits limitations in separating proteins and other biopolymers, as well as small highly hydrophobic molecules. The poor selectivity of MECC for the separation of proteins has been attributed to several factors including the strong interactions between the hydrophobic domains of the protein molecules and the micelles, the inability of large biopolymers to penetrate into the micelles and the binding of the monomeric surfactant to the proteins. The result is that even though the surfactant concentration in the

[15] Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. *Anal. Chem.* **1984**, *56*, 111-113.
[16] Khaledi, M.G. Micellar Electrokinetic Capillary Chromatography. In *Handbook of Capillary Electrophoresis*; Landers, J.P., Ed.; CRC Press: Boca Raton, FL, **1993**; Chapter 3.
[17] Terabe, S.; Otsuka, K.; Kawahara, J.; Tatekawa, K. *J. Chromatogr.* **1991**, *559*, 209-214.
[18] Otsuka, K.; Terabe, S. *J. Chromatogr.* **1990**, *515*, 221-226.
[19] Terabe, S.; Shibata, M.; Miyashita, Y. *J. Chromatogr.* **1989**, *480*, 403-411.

electrolyte solution exceeds the critical micelle concentration, the protein-surfactant complexes are likely to be not subjected to partition in the micelles as do smaller molecules.[20]

[20] Corradini, D. *J. Chromatogr B*, **1997**, *699*, 221-256.

# Chapter 3-*Liposome Capillary Electrophoresis of Peptides and Proteins*

## *3.1 Introduction*

Phospholipid liposomes have been largely employed as an appropriate model to study membrane properties and interactions, for example interactions between lipoproteins and drugs or other small molecules, peptide/proteins interactions, channel formation and transport of molecules across cellular interfaces.[1] Many investigations with several techniques have been carried out on the factors that influence the interactions of vesicles with biologically active small compounds and macromolecules. Dynamic Light Scattering measurements,[1a] viscosity and turbidity measurements as well as Raman Resonance, Circular Dichroism and UV-vis absorption spectroscopy,[1b,1c] Atomic Force Microscopy,[1d] NMR spectroscopy[1e,1f] and Surface Plasmon Resonance[1g] were useful in the investigation on the membrane models interactions of peptides and proteins. Recently, investigations on the chemical/physical affinity of biologically active compounds for membrane models have been carried out by CE, indicating a new exploitation of the technique previously addressed mainly to analytical separations.[2] The dynamically flowing environment that capillary electrophoresis offers concerning the interactions of lipid vesicles with biologically active molecules, such as drugs, proteins or peptides, can be very indicative of transport through membranes and can provide insight into the physiological in vivo behaviour of these bioactive compounds.

In this chapter the characterization of liposomes composed by the zwitterionc phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and the influence of the presence of a liposome dispersion in bare-fused silica capillaries on the electro-osmotic flow and on the electrophoretic behaviour of closely related peptides and basic proteins will be described. Aim of this work is exploring the suitability of liposome capillary electrophoresis for investigating the interactions between liposomes

[1] (a) Bergers, J.J.; Vingerhoeds, M.H.; van Bloois, L.; Herron, J.N.; Janssen L.H.M.; Fischer, M.J.E.; Crommelin, D.J.A. *Biochemistry*, **1993**, *32*, 4641-4649. (b) Oellerich, S.; Leconte, S.;Paternostre, M.; Heimburg, T.; Hilebrandt, P. *J. Phys. Chem. B* **2004**, *108*, 3871-3878. (c) Persson, D.; Thorén, P.E.G.; Herner, M.; Lincoln, P.; Nordén, B. *Biochemistry*, **2003**, *42*, 421-429. (d) You, H.X.; Qi, X.; Grabowski, G.A.; Yu, L. *Biophys. J.* **2003**, *84*, 2043-2057. (e) Mason, J.A.; Lopez, J.J.; Beyermann, M.; Galubitz, C. *Biochim. Biophys. Acta* **2005**, *1714*, 1-10. (f) Vogel, A.; Scheidt, H.A.; Huster, D. *Biophys. J.* **2003**, *85*, 1691-1701. (g) Papo, N.; Shai, Y. *Biochemistry*, **2003**, *42*, 458-466.

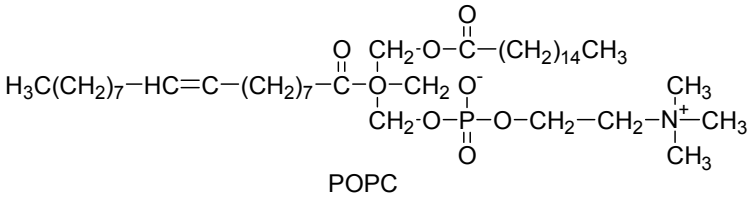
[2] Zhang, Y.; Zhang, R.; Hjerten, S.; Lundhal, P. *Electrophoresis* **1995**, *16*, 1519-1523. Manetto, G.; Bellini, M.S.; Deyl, Z. *J. Chromatogr. A* **2003**, *990*, 205-214.

and biomolecules and for separating analytes which display similar electrophoretic behaviour or strongly interact with the inner capillary wall of the bare-fused silica capillaries. The investigation will be performed with a liposome dispersion of unilamellar vesicles extruded at 100 nm.

## *3.2 Results and Discussion*

### *3.2.1 Characterization of Extruded Phospholipid Liposomes*

The homogeneity and distribution size of the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes dispersed in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl were investigated by capillary electrophoresis (CE) and by dynamic light scattering (DLS).




The electropherogram displayed in Figure 1 shows that the POPC peak exhibited a consistent Gaussian shape, indicating homogeneous and relatively narrow size distribution of the liposomes in accordance with the dynamic light scattering measurements which showed an average hydrodynamic diameter of 126±0.3 nm and polydispersity 0.087±0.022 of the POPC dispersion (Figure 2).

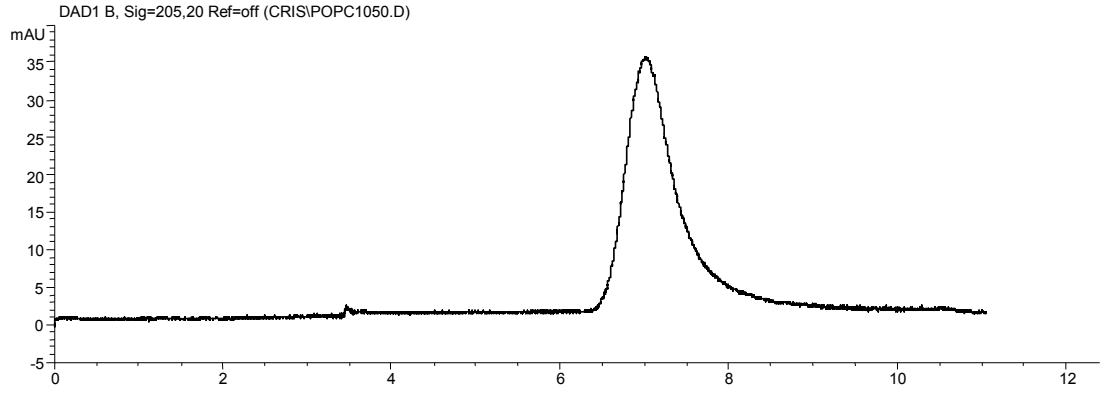



**Figure 1.** Electrophoretic behaviour of POPC liposome dispersion. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 205 nm; sample 8.0 mM POPC extruded at 100 nm, in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl.

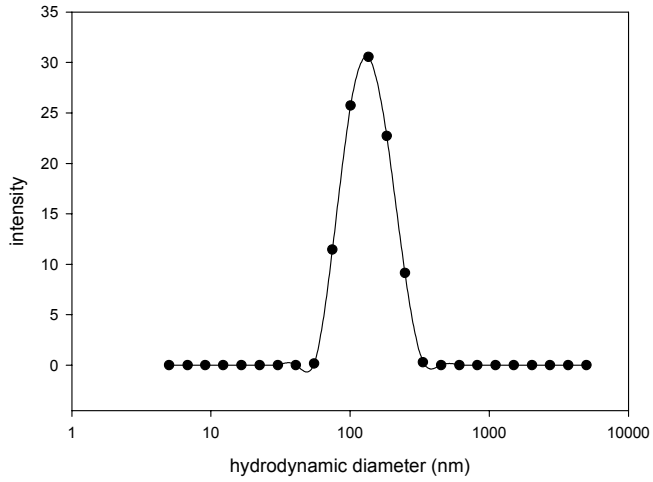



**Figure 2.** Semi-logarithmic plot of dynamic light scattering measurements of a 0.4 mM POPC 100 nm dispersion.

Both CE and DLS show a narrow distribution indicating a monodispersed solution of LUV. The expected size dependent electrophoretic migration behavior of vesicles[3] indicate CE a possible alternative to laser light scattering for monitoring liposome preparation quality.[4] After preconditioning the capillary with the electrolyte solution, the electro-osmotic flow was measured on the basis of the migration time of the neutral

[3] (a) Radko, S.; Chrambac, A. *J. Chromatogr. A* **1999**, *722*, 1-10. (b) Roberts, M.A.; Lo Cascio-Brown, L.; MacCrehan, W.A.; Durst, R.A. *Anal Chem*. **1996**, *68*, 3434–3440.
[4] (a) Radko, S.; Stastna, M.; Chrambac, A. *Anal. Chem.* **2000**, *72*, 5955-5960. (b) Radko, S.; Stastna, M.; Chrambac, A. *J. Chromatogr. B* **2001**, *761*, 69-75.

marker mesityl oxide. Repeated injections resulted in an average migration time for mesityl oxide of 3.17 min (SD, 0.01; RSD, 0.39%), which corresponded to an electro-osmotic mobility of $4.25 \times 10^{-8}$ $m^2V^{-1}s^{-1}$. Multiple injections of POPC dispersion sample resulted in an average migration time for POPC liposomes of 7.41 min (SD, 0.4 min; RSD, 5.60%), which corresponded to an average apparent mobility of $1.82 \times 10^{-8}$ $m^2V^{-1}s^{-1}$. This indicated that POPC liposomes exhibit a slightly negative charge and migrate against the electro-osmotic flow, in accordance with the negative surface charge of POPC liposomes evidenced by surface potential measurements[5] and capillary electrophoresis.[6]

### *4.2.1 Influence of Liposomes on the Electro-osmotic Flow*

Because liposomes are expected to interact with the inner surface of bare fused-silica capillaries with the result of influencing the electro-osmotic flow,[3a,7] we investigated the effect on the electro-osmotic flow of POPC liposomes dispersed in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl introduced into the capillary as plugs of different volumes. The study was performed by injecting POPC liposomes plugs of different volumes and measuring the electro-osmotic flow after each plug injection. The values of the coefficient of the electro-osmotic flow plotted as a function of the volume of POPC liposome dispersion plug are displayed in Figure 3.

[5] Makino, K.; Yamada, T.; Kimura, M.; Oka, T.; Ohshima, H.; Kondo, T. *Biophys. Chem*. **1991**, *41*, 175-183.
[6] Wiedmer, S.K.; Hautala, J.T.; Holopainen, J.M.;Kinnunen, P.J.K.; Riekkola, M.L. *Electrophoresis* **2001**, *22*, 1305-1313.
[7] Hautala, J.T.; Linden, M.V.; Wiedmer, S.K.; Ryhanen, S.J.; Saily, M.J.; Kinnunen, P.J.K.; Riekkola, M.L. *J. Chromatogr. A* **2003**, *1004*, 81-90.

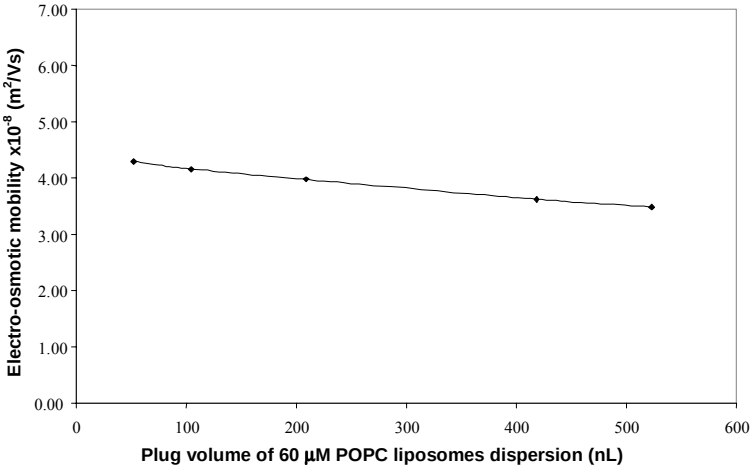



**Figure 3.** Effect of plug volume of a 60 µM POPC liposomes dispersed in the BGE on the electro-osmotic flow. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm.

The shape of this curve clearly shows that the EOF decreases with increasing the volume of the POPC plug. Rinsing the capillary tube with a volume of the 60 µM POPC liposome dispersion, corresponding to five times the volume of the capillary tube, resulted in decreasing the electro-osmotic flow down to $9.08 \times 10^{-9}$ $m^2V^{-1}s^{-1}$, which is about one order of magnitude lower than the value measured in absence of POPC liposome. Similar results were obtained by rinsing the capillary with the POPC liposome dispersion by different rinsing times ranging from 5 to 20 min, all exceeding the time requested for replacing one volume of the capillary column. This indicates that the influence of POPC liposomes on the electro-osmotic flow is significantly greater when the contact zone of the liposome dispersion with the inner wall of the capillary tube is extended to the whole length of the capillary and it is protracted for a given time period. Nevertheless, no appreciable variations in the electro-osmotic flow were noted by rinsing the capillary with the POPC liposome dispersion for times longer than 20 min, indicating that an equilibrium state is reached within this time period. Removing the POPC liposomes plug by rinsing the capillary with the electrolyte solution for 15 min caused the increasing of the coefficient of the electro-osmotic mobility to $3.99 \times 10^{-8}$ $m^2V^{-1}s^{-1}$, which was only 6.1% lower than the average value measured before rinsing the capillary with POPC liposome. The electro-osmotic flow was almost restored to its original value by rinsing the capillary with the electrolyte solution for additional 15 min, indicating a rather poor stability of the dynamic coating of the capillary wall

obtained by rinsing the capillary tube with the POPC liposomes dispersed in the 20mM phosphate buffer, pH 7.4, containing 68mM NaCl. These findings are in agreement with the result of a recent study on the use of liposomes as dynamic coating agents for bare fused silica capillaries employed for the separation of steroids; this in fact reports poor stability of the dynamic coating obtained with a POPC liposome dispersion in phosphate buffer.[7]

The effect on the electro-osmotic flow of rinsing the capillary tube with liposome dispersion of different concentration of POPC liposomes was studied. The study was conducted by rinsing sequentially the capillary with a liposome dispersion containing increased concentration of POPC liposomes, ranging from 30 to 200 μM. After each 20 min rinsing time we measured the electro-osmotic flow with the capillary tube filled with the liposome dispersion. The dependence of electro-osmotic flow from the concentration of POPC liposomes is displayed in Figure 4 as a plot of the coefficient of the electro-osmotic flow versus the concentration of the liposome.

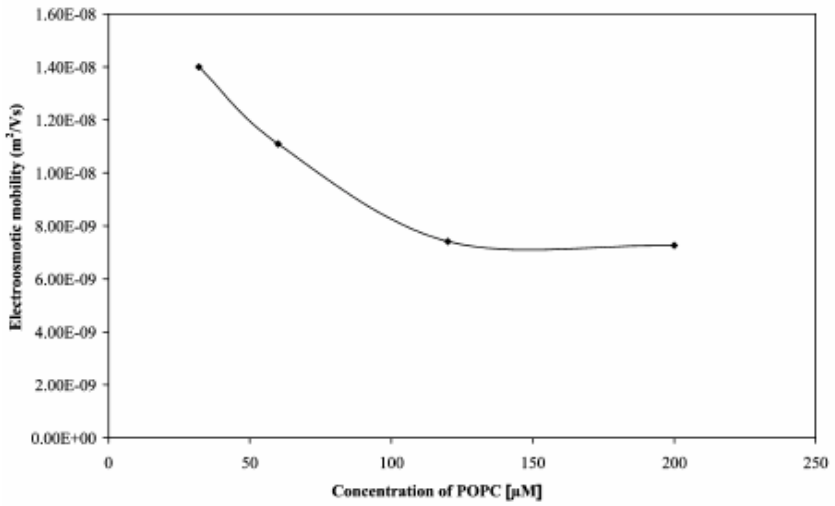



**Figure 4.** Effect of the concentration of POPC liposomes dispersed in the BGE on the electro-osmotic flow. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm.

The electro-osmotic flow decreases by increasing the concentration of POPC liposomes and the curve describing the dependence of the coefficient of the electro-osmotic flow from the concentration of POPC liposomes has a ‘‘Langmuir shape’’, suggesting that the observed variation is the result of the liposomes adsorption in the Stern region of the electric double layer at the interface between the capillary wall and the electrolyte

solution, as in the case of a variety of oligoamines.[8] These compounds have been found to adsorb at the interface between the capillary wall and the electrolyte solution, determining the reduction and, in some cases, the reversal of the direction of the electro-osmotic flow. The dependence of the electro-osmotic flow on the concentration of positively charged adsorbing compounds (C) incorporated in the electrolyte solution has been described by a theoretical model that correlates the electro-osmotic mobility to the charge density in the Stern region of the electric double layer (arising from the adsorption of the charged compounds) and to the charge density at the capillary wall (resulting from the ionization of silanol groups).[9]

According to this model, the dependence of the electro-osmotic mobility on the concentration of the adsorbing compound (C) in the electrolyte solution is expressed as

$$\mu_{eo} = \frac{\kappa^{-1}}{\eta}\left[\frac{zen_0\frac{C}{V_m}\exp\left(\frac{ze\psi_\delta + \Phi}{kT}\right)}{1+\frac{C}{V_m}\exp\left(\frac{ze\psi_\delta + \Phi}{kT}\right)} - \left(\frac{\gamma}{1+\frac{[H^+]}{K_a}}\right)\right]$$

where $\kappa^{-1}$ is the Debye-Hückel thickness of the diffuse double layer, $\eta$ is the viscosity of the electrolyte solution, *e* is the elementary charge, *z* is the valence of the adsorbing ion, k is the Boltzman constant, T is the absolute temperature, $n_O$ is the number of accessible sites in the Stern layer, $V_m$ is the molar volume of the solvent, $\Phi$ is the specific adsorption potential of the adsorbing compound, $\gamma$ is the sum of the ionized and protonated surface silanol groups, $[H^+]$ is the bulk electrolyte hydrogen ion concentration, and $k_a$ is the silanol dissociation constant. According to this equation, at constant pH and conditions, where the variations of ionic strength and viscosity are irrelevant, the electro-osmotic mobility depends mainly on the surface density of the adsorbed compounds in the Stern region of the electric double layer, which follow a Langmuir-type adsorption model. This prediction has been shown to be in good agreement with the experimental electro-osmotic mobility trends measured with several

[8](a) Corradini, D.; Cannarsa, G. *Electrophoresis* **1995**, *16*, 630-635. (b) Corradini, D.; Sprecacenere, L. *Chromatographia* **2003**, *58*, 1-10. (c) Corradini, D.; Cogliandro, E.; D'Alessandro, L.; Nicoletti, I. *J. Chromatogr. A* **2003**, *1013*, 221-232.

[9] Corradini, D.; Rhomberg, A.; Corradini, C. *J. Chromatogr. A* **1994**, *661*, 305-313.

additives at different concentrations[9,10] and pH values[8a] and it seems to be adaptable to describe the variation of electro-osmotic flow with the concentration of dispersed liposomes into the electrolyte solution.

### *3.2.3 Liposome Capillary Electrophoresis of Peptides*

Experiments were performed with the purpose of evaluating the influence of POPC liposomes on the electrophoretic migration behaviour of a model peptides in order to evaluate the suitability of liposome capillary electrophoresis both for investigating the selective and specific interactions between the aggregates and the peptides and hence the potentiality of CE at separating analytes exhibiting similar electrophoretic behaviour. Three synthetic peptides of formula weight ranging from 931.11 to 1296.49 Da and amino acid sequence of human angiotensin I, angiotensin II and angiotensin III, whose primary structure varies from one to three amino acids, were employed. Angiotensin I is a putative neurotransmitter while angiotensin II is important in regulating cardiovascular hemodynamics and cardiovascular structure. However, the three angiotensins were selected as model peptides because of their closely related primary structure and consequent similar electrophoretic behaviour which make their resolution critical without the incorporation of a suitable additive into the electrolyte solution (see below).

A first set of experiments were carried out by injecting the peptide mixtures after a partial filling of the capillary with a plug of increasing volume of the POPC liposome dispersed in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl (Figure 5).

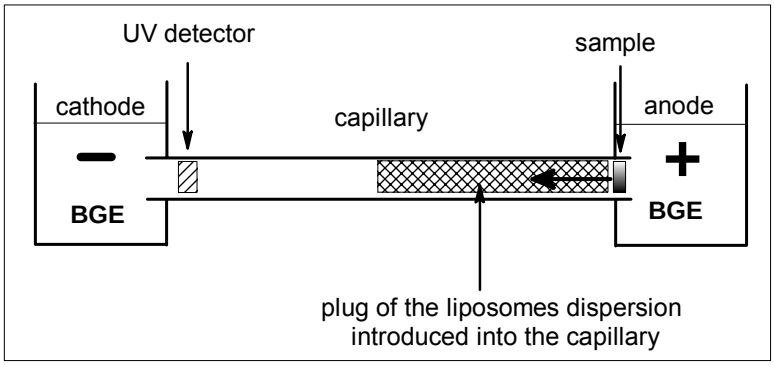



**Figure 5.** Schematic illustration of the plugs of the liposome dispersion introduced into the capillary. The liposome dispersion was introduced by pressure into the capillary from the anodic end. Samples cross the plug during their migration toward the cathodic end interacting with the liposomes dispersed in the plug.

[10] Corradini, D.; Cannarsa, G.; Fabbri, E.; Corradini, C. *J. Chromatogr. A* **1995**, *709*, 127-134.

Figure 6 shows the electrophoretic behaviour of the three closely related peptides in the absence and in the presence of a plug of POPC liposome dispersion introduced into the capillary.

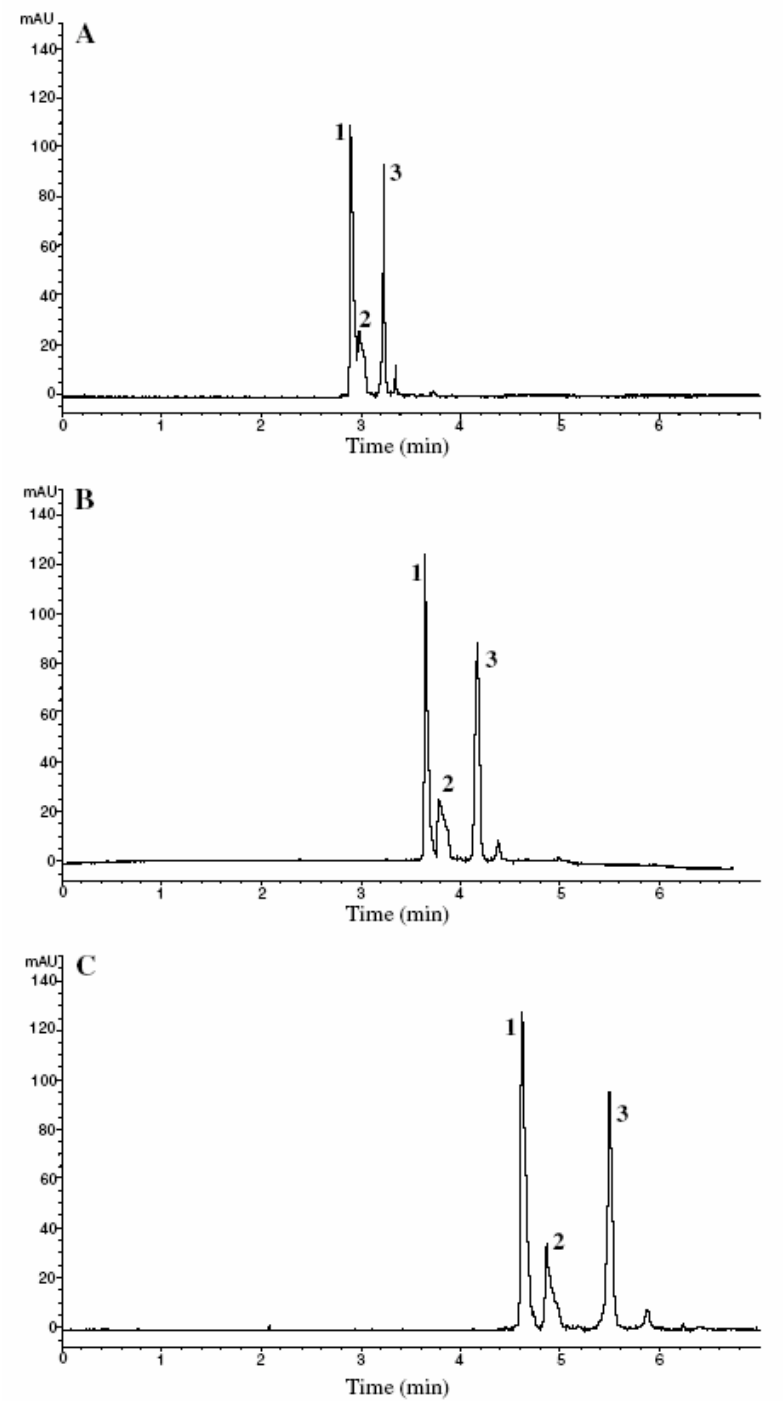



**Figure 6.** Electrophoretic behaviour of model peptides in the absence (A) and in the presence (B–C) of POPC liposome dispersion in the capillary tube. Capillary partially filled with 250 nL (B) and 500 nL (C) of 200 μM POPC liposomes dispersed in 20mM phosphate buffer, pH 7.4, containing 68mM NaCl; Peptides, 1=angiotensin III, 2=angiotensin II, 3=angiotensin I. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm.

It can be observed that the migration times of peptides and their resolution increased with increasing the volume of the plug of the liposome dispersion introduced into the capillary tube. To evaluate the influence of the liposome concentration on the electrophoretic behaviour of the peptides, the capillary was completely filled with a POPC liposome dispersion of increasing concentrations in 20mM phosphate buffer, pH 7.4, containing 68 mM NaCl. Figure 7 shows a slightly increase in the migration times and resolution of the model peptides for a 6 μM concentration of POPC liposome, and a significantly larger effect when the capillary is completely filled with a solution of 200 μM of POPC liposome dispersion.

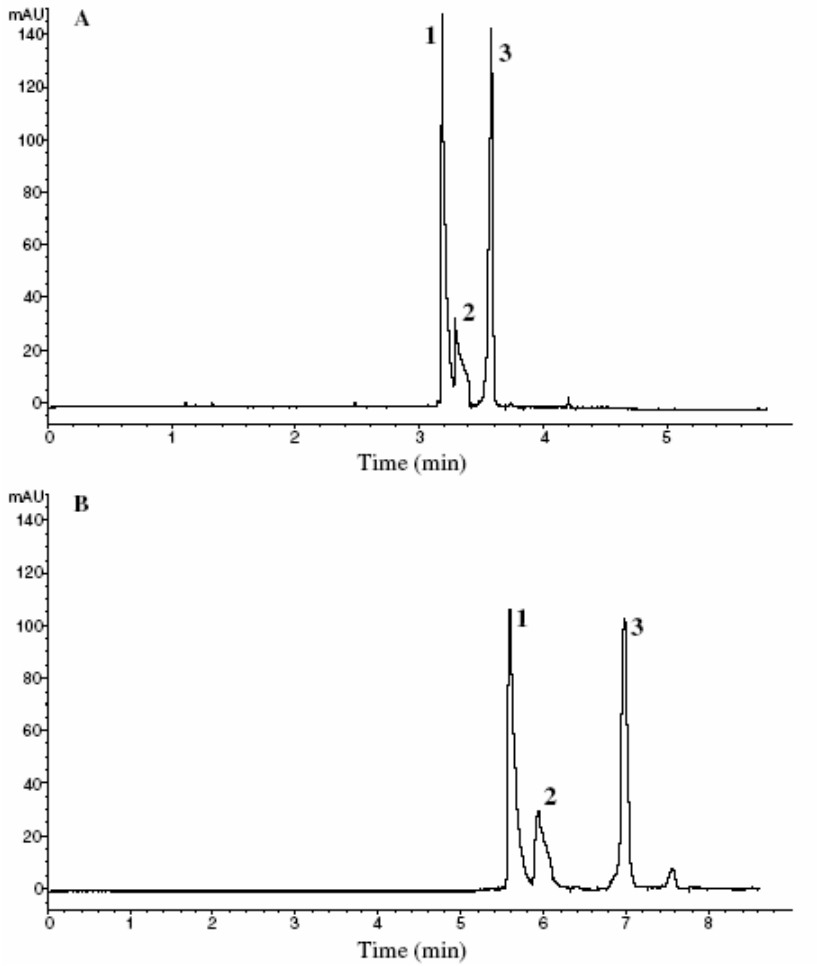



**Figure 7.** Electrophoretic behaviour of model peptides in the presence of POPC liposomes of increasing concentrations in the capillary tube. Capillary completely filled with 6.0 μM (A) and 200 μM (B) POPC liposomes dispersed in 20mM phosphate buffer, pH 7.4 containing 68mM NaCl. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm. Peptides, 1=angiotensin III, 2=angiotensin II, 3=angiotensin I.

The results of repeated runs of the peptides mixture carried out to evaluate the repeatability of migration times are reported in Table 1.

**Table 1.** Repeatability of migration times of three model peptides in LCE with the capillary completely filled with 200 μM POPC liposomes dispersed in 20mM phosphate buffer, pH 7.4 containing 68mM NaCl.

| Run | Migration Time (min) | | |
|---|---|---|---|
| | Angiotensin I | Angiotensin II | Angiotensin III |
| 1 | 5.43 | 5.77 | 6.71 |
| 2 | 5.86 | 6.26 | 7.42 |
| 3 | 5.84 | 6.21 | 7.36 |
| 4 | 5.59 | 5.93 | 6.99 |
| <tm> | 5.68 | 6.04 | 7.12 |
| SD | 0.18 | 0.20 | 0.29 |
| RSD (%) | 3.1 | 3.3 | 4.0 |

The results show that both the volume of the liposome plug and the concentration of POPC liposomes in the plug influence the migration behaviour of the peptides. In particular, the migration times of peptides increase by increasing either the volume of the liposome plug or the concentration of POPC liposomes in the plug. It could be expected that the observed variations in the migration times are the result of interactions between liposomes and peptides. On the other hand, many other factors may contribute to the observed variations in the retention times of the investigated peptides, including changes in the electro-osmotic flow, viscosity of the BGE and of the POPC liposome plug, temperature effects, interactions with the capillary wall. Relative mobilities, or selectivity, of the three peptides as a function of the volume of liposome plugs containing 200 μM POPC liposomes are reported in Figure 8. These plots suggest that the observed variations in selectivity should be the results of specific interactions of peptides with POPC liposomes rather than the effect of the variations in parameters that are expected to contribute indifferently to the mobility of samples, such as the electro-osmotic flow or the viscosity of the liposome dispersion. For example, angiotensin II and angiotensin III are very close in size and, thus, are expected to display similar behaviour by changing the viscosity. Similarly, the possible variations in the electro-osmotic flow are expected to contribute indifferently to the mobility of the three peptides. Conversely, plots of Figure 8 show different variations in selectivity depending on the volume of POPC liposomes plug and peptide pair. The relative mobility of the peptide pair angiotensin II / angiotensin I is only little affected at small volumes of POPC liposomes plug whereas it increases significantly as the volume of POPC liposomes plug become larger than 500 nL. A similar behaviour is also observed for the other peptide pair, and the different slope of the two curves, describing the

variation of selectivity with the volume of POPC liposomes plug, further corroborate the action of selective interactions between the peptides and the liposomes dispersed into the plug.

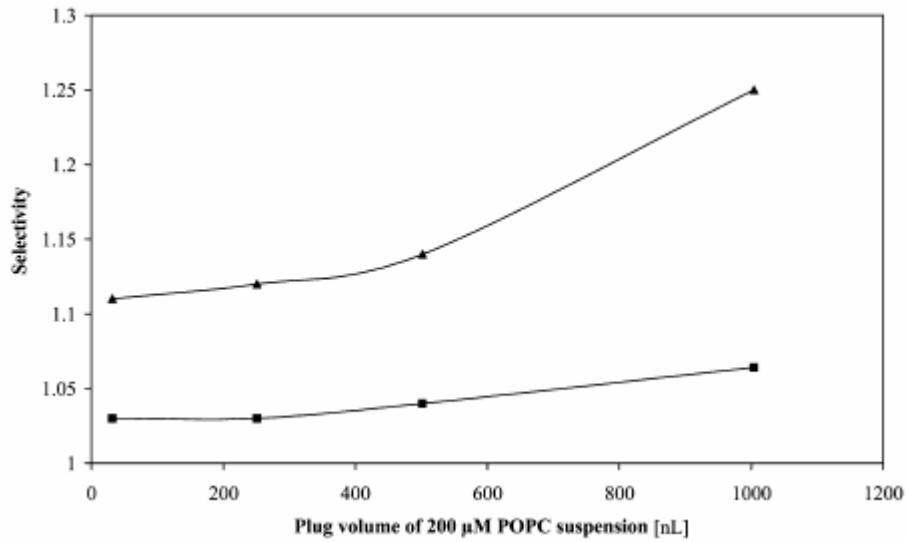



**Figure 8.** Effect of plug volume of a 200 µM POPC liposomes dispersed in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl solution on selectivity of model peptides (apparent mobility ratio of (■) angiotensin II/angiotensin III and (▲) angiotensin I/angiotensin II). Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm.

Our experiments (section 3.2.1.) together with literature data[5,6] indicate that POPC liposomes exhibit negative net charge and, therefore, are expected to establish electrostatic interactions with the positively charged amino acid residues of the model peptides. At pH 7.4 angiotensin III, whose isoelectric point (pI) is 8.75,[11] is positively charged and is expected to interact with the negative surface charge of the POPC liposomes. In addition, its sequence (section 3.4.5) shows the presence of two basic residues, hystidine and arginine positively charged at pH 7.4, that can further influence the electrostatic interactions of angiotensin III with the net negative charge featured by the outer surface of the POPC liposomes. Angiotensin I and angiotensin II are expected to exhibit similar net negative charge at pH 7.4, according to their isoelectric points, which are very close to each other, being 6.92 and 6.74 respectively. In spite of these apparently unfavourable conditions to establish selective electrostatic interactions with the POPC liposomes, which also exhibit a net negative charge at pH 7.4, these peptides

[11] The isoelectric points (pI) of the angiotensi I, II and III were calculated at ExPASy Proteomic Server: www.expasy.org/tools/pi_tool.html.

show the larger variations of relative mobility (i.e., selectivity) by increasing the volume of POPC liposomes dispersion into the capillary tube. The primary sequence of angiotensin I and angiotensin II comprises two basic residues of hystidine and arginine, that may establish electrostatic interactions with the net negative charge of the POPC liposomes. Moreover, the acidic residue aspartic acid, present in the amino acidic sequence of angiotensin I and angiotensin II could interact with the positively charged choline residue, probably buried into the internal region surface of the POPC liposomes. Finally, angiotensin I differs from angiotensin II for additional hystidine and leucine residues, which may influence the capability of this peptide of interacting with the POPC liposomes by both electrostatic and hydrophobic interactions. Therefore, these observations are in accordance with the expectation that the interactions of the model peptides with liposomes are affected by the different amino acid residues of the peptide; these may in fact establish both electrostatic and hydrophobic interactions with the outer surface of liposomes, rather than being the result of the action of their net electrostatic charge.

### *3.2.4 Liposome Capillary Electrophoresis of Basic Proteins*

Capillary electrophoresis has been widely employed by a variety of modes in the analysis of proteins.[12] Each mode employs the same instrumentations but is different in the separation mechanism promoting the differential migration of analytes under the action of an electric field. In Capillary zone electrophoresis (CZE) the velocity of migration is proportional to the electrophoretic mobility of the analytes, which depends on their effective charge-to-hydrodynamic radius ratios. This mode of capillary electrophoresis results to be the simplest and probably the most commonly employed mode of CE for the separation of proteins. Nevertheless, the molecular complexity of proteins requires particular attention in selecting the capillary tube and, in particular, the composition of the solution employed as the background electrolyte.

Proteins are ionogenic substances also bearing other functionalities as hydrogen-bonding regions, hydrophobic patches and biospecific interaction sites that can interact to different extents with the other constituents of the sample mixture and with the components of the electrolyte solutions used for their separation by CE.

[12] (a) Stutz, H.; *Electrophoresis* **2005**, *26*, 1254-1290. (b) Dolnik V.; Hutterer K.M.; *Electrophoresis* **2001**, *22*, 4163-4196. (c) Dolnik V. *Electrophoresis* **1999**, *20*, 3106-3115.

Depending on the chemical nature of both the protein and the ionic species in solution these interactions can lead to significant variations in the electrophoretic mobilities of proteins and can greatly affect their selective separation by capillary electrophoresis. In addition, the different functionalities of proteins exposed to the aqueous solution may interact with a variety of active sites on the inner surface of the fused-silica capillaries; these comprise inert siloxane bridges, hydrogen-bonding sites and different types of ionisable silanol groups (vicinal, terminal and isolated).[13] These interactions may give rise to peak broadening and asymmetry, irreproducible migration times, low mass recovery and in some cases irreversible adsorption. Many approaches to overcome the detrimental effects of protein-capillary wall interactions are traditionally employed.[14] One of them is to realize a permanent coating of the inner surface of the capillaries with a neutral hydrophilic compound. A variety of alkylsilane, carbohydrates and neutral polymers have been covalently bonded to the silica capillary wall by silan derivatization.[15] However, hydrogen-bonding and hydrophobic interactions may also occur in chemically modified capillaries which often suffer from a gradual loss of surface coverage, particularly at high pH values. Other strategies consist in the use of electrolyte solutions at extreme pH values, whether acidic, to suppress the silanol dissociation, or higher than protein pI to have both the protein and the capillary wall negatively charged. Other possibilities are represented by the use of electrolyte solutions with high concentrations of alkali salts or zwitterions, to suppress the electrostatic interactions between protein and the capillary wall, and by the application of an applied radial voltage to the capillary to lower the zeta potential. There are several disadvantages of the above strategies. First, extremes pH tend to denature proteins and compromise recovery by reducing solubility. In addition, the current derived from ionic strength limits the voltage that can be applied, with concomitant decrease in efficiency and increase in the analysis times. Finally, the option of controlling zeta potential by an external electric field is not available on commercial units. Another approach is to incorporate a suitable additive into the running electrolyte solution. Most of the additives act either as masking or competitive agents for the silanol groups on the inner

[13] Unger, K.K. *Porous Silica: Its properties and Use as Support in Column Liquid Chromatography*, Elsevier, Amsterdam, **1979**.
[14] Corradini, D. *J. Chromatogr. B*. **1997**, *699*, 221-256.
[15] Horvath J.; Dolnik, V. *Electrophoresis* **2001**, *22*, 644-655.

wall of the capillary, so that they are not accessible to protein interactions. Other additives may function as strong ion-pairing or competitive agents for the basic amino acid residues of the proteins exposed to the electrolyte solution, in order to subtract their availability to the silanol groups on the capillary wall. Additives may also be introduced to the electrolyte solutions to improve the selectivity by interacting specifically or to different extents with the proteins in the sample and to extend the application of CE to study specific non-covalent interactions between proteins and organic or inorganic compounds, either charged or neutral.

On the frame of the investigation on the interactions between liposomes and biopolymers, four water soluble basic proteins, namely lysozyme, cytochrome C, ribonuclease A and α-chymotrypsinogen A, with isoelectric points ranging from 9.5 (cytochrome C) to 11.0 (lysozyme) and molecular weight comprised from 12400 (cytochrome C) to 25000 Da (α-chymotrypsinogen A),[16] were taken into consideration. The four basic proteins strongly interact with the inner surface of bare fused capillaries in the wide pH range at which the proteins are positively charged and the silanol groups on the capillary wall ionized, i.e. at any pH value higher than 2. Hence, liposome capillary electrophoresis was performed in order to evaluate the suitability of capillary electrophoresis to be useful both for the comprehension of the molecular interactions between liposomes and basic proteins and for separating basic proteins in acidic domain using bare-fused silica capillaries. In general water soluble proteins exhibit a hydrophilic surface constituted mainly by polar and charged amino acid side chains and the interactions between proteins and liposomes are expected to be mediated by electrostatic and/or by hydrophobic forces.[1a,1b,17]

At pH 7.4 the four basic proteins are positively charged and owing to the strong interactions with the negatively charged inner surface of the bare-fused silica capillary, proteins do not migrate as discrete peaks (Figure 9).

[16] Righetti, P.G.; Caravaggio, T. *J. Chromatogr*. 1**976**, *127*, 1-28.
[17] Zschornig, O.; Paasche, G.; Thieme, C.; Korb, N.; Arnold, K. *Coll. Surf. B: Biointerfaces* **2005**, *42*, 69-78.

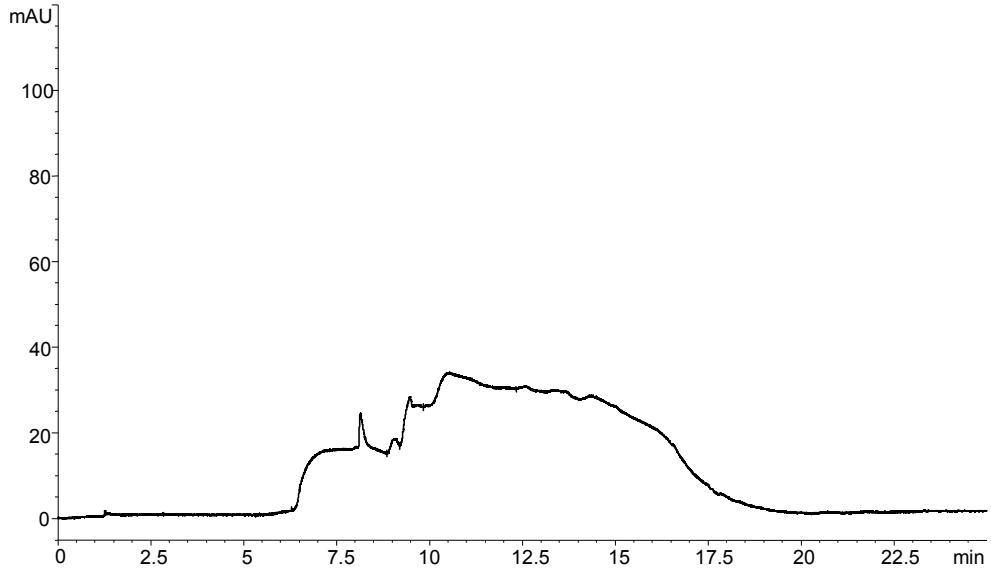



**Figure 9.** Electrophoretic behaviour of the four basic proteins (lysozyme, cytochrome C, ribonuclease A and α-chymotrypsinogen A) in bare fused silica capillary in the absence of a POPC liposome dispersion into the capillary tube. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm.

Complete separation of the basic proteins was observed upon filling the capillary with 60 μM POPC dispersed in, 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl which was also employed as electrolyte solution (Figure 10).

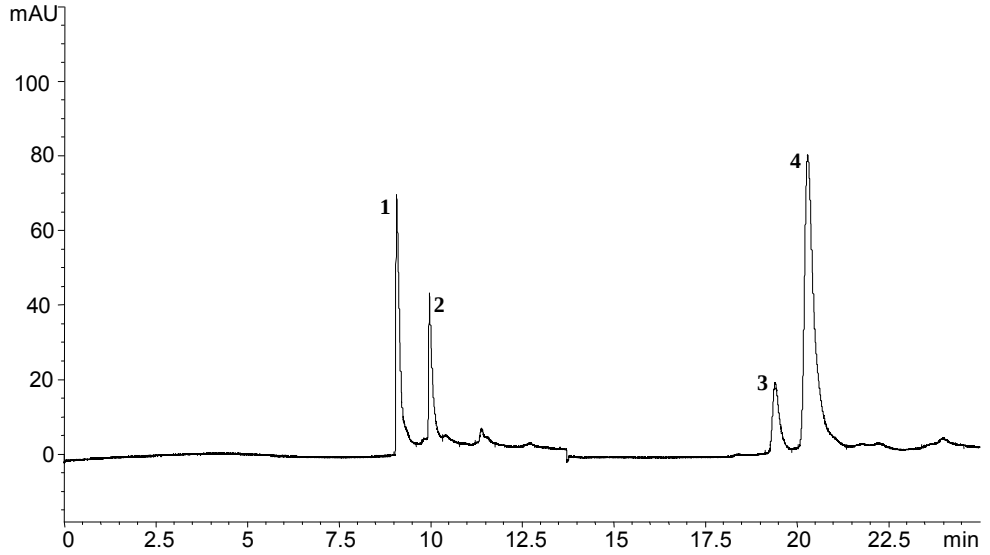



**Figure 10.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillaries filled with 60 μM POPC liposomes dispersed in 20mM phosphate buffer, pH 7.4, containing 68mM NaCl, which was also employed as the electrolyte solution (BGE). Proteins, 1=lysozyme, 2=cytochrome c 3=ribonuclease A, 4=α-chymotrypsinogen A. Applied voltage, 10.0 kV; cathodic detection at 214 nm.

The variation of the electrophoretic behaviour of the four basic proteins upon incorporating the liposome dispersion into the electrolyte solution employed to fill the capillary tube and the electrolyte reservoirs can be explained as following. The positively charged proteins are expected to interact with the capillary wall and the liposome surface, which are both negatively charged. However, the inner capillary surface exposed to the electrolyte solution is expected to be considerably lower than the total surface area of the dispersed liposomes into the capillary tube. Hence, the untoward interactions between basic proteins and the capillary wall, promoting the observed irreversible adsorption of these biopolymers at pH 7.4 in absence of POPC (Figure 9), are believed to be minimized by the competitive interactions that the basic proteins may establish with the liposomes dispersed in the electrolyte solution. The need of incorporating POPC into the background electrolyte was probably due to the instability of the POPC coating on the inner capillary wall; in this way liposomes migrating from the inner capillary wall toward the cathode are restored by the liposomes migrating from anodic compartment to cathode.

### *3.2.5 Liposome Capillary Electrophoresis of Basic Proteins: Influence of pH and Composition of BGE*

The study was conducted using liposomes dispersed in various buffer solutions of different composition and pH values ranging from pH 6.2 to 4.0 by two different experimental approaches (as described in section 3.4).

*Experiments at pH 6.2*.

Figure 11 shows the electrophoretic run of the basic proteins mixture carried out with the liposome dynamically coated capillary either in the presence (panel A) or in the absence (panel B) of POPC dispersed in 20 mM phosphate buffer, pH 6.2 containing 68 mM NaCl (BGE) filled into the capillary.

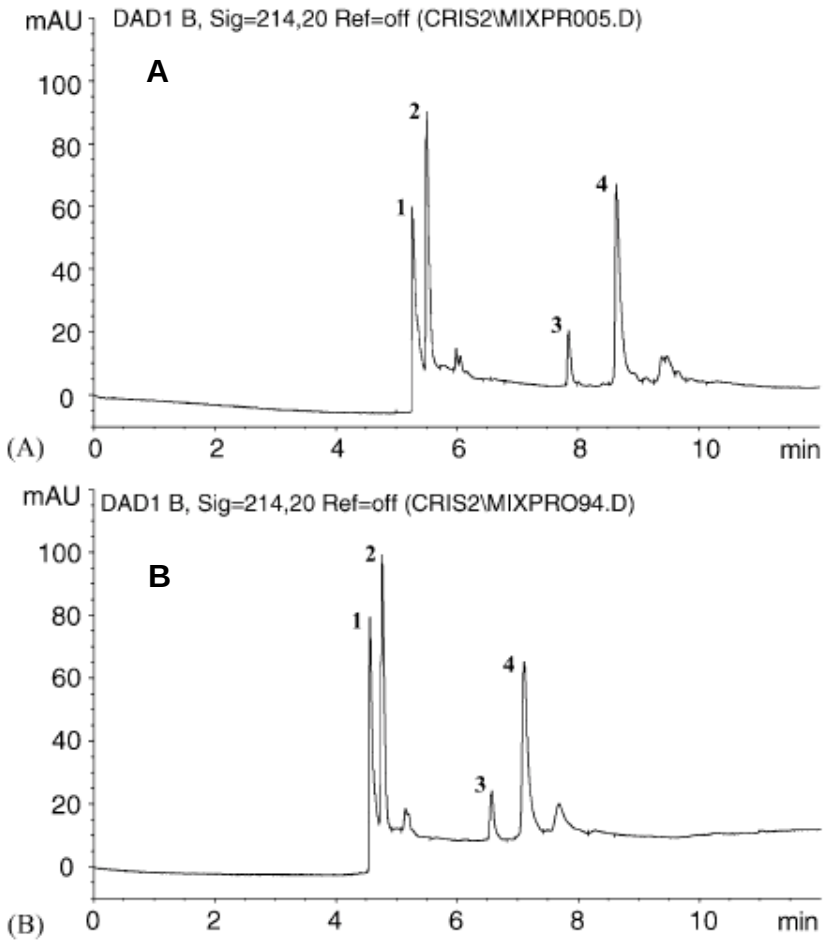



**Figure 11.** Electrophoretic behaviour of basic proteins at pH 6.2 in POPC dynamically coated capillary either filled with 60 μM POPC liposomes dispersed in BGE (A) or with BGE (POPC-free BGE) (B). Electrolyte solution (BGE), 20mM phosphate buffer, pH 6.2, containing 68mM NaCl; applied voltage, 10.0 kV; cathodic detection at 214 nm. Proteins: (1) lysozyme, (2) cytochrome *c*, (3) ribonuclease A, and (4) α-chymotrypsinogen A.

It can be seen that both approaches allowed the migration of basic proteins as discrete peaks, that otherwise were irreversibly adsorbed onto the capillary wall of the bare fused-silica capillary when electrophoresis was carried out with phosphate buffer at pH 6.2 without dynamically coating or filling the capillary with POPC liposomes. In addition, it clearly appears that the presence of POPC liposomes dispersed in the buffer solution filled into the capillary causes an improvement of resolution, selectivity and peak shape of the two completely separated proteins (i.e. ribonuclease A and α-chymotrypsinogen A) at the expenses of increased migration time (Table 2 and 3).

**Table 2.** Effect of POPC liposomes dispersed in the BGE filled into the capillary on resolution using either bare-fused-silica or POPC dynamically coated capillaries

| pH | Capillary | [POPC] (μM) | Resolution | |
|---|---|---|---|---|
| | | | Lys/Cyt | Rnase/Chy |
| 6.2 | POPC-coated | 60 | 1.70 | 11.54 |
| 6.2 | POPC-coated | 0[a] | 3.16 | 7.04 |
| 5.2 | POPC-coated | 60 | 1.71 | 6.71 |
| 5.2 | POPC-coated | 0[a] | 1.91 | 2.73 |
| 6.0 | Not dynamically coated | 120 | 3.70 | 1.03 |
| 5.0 | Not dynamically coated | 120 | 1.51 | 3.30 |
| 4.0 | Not dynamically coated | 120 | 6.33 | 9.88 |

Lys: lysozyme; Cyt: cytochrome C; Rnase: ribonuclease A; Chy: α-chymotrypsinogen A.
[a] To indicate capillary filled with POPC-free BGE.

**Table 3.** Effect of POPC liposomes dispersed in the BGE filled into the capillary on efficiency using either bare-fused-silica or POPC dynamically coated capillaries

| pH | Capillary | [POPC] (μM) | Efficiency | | | |
|---|---|---|---|---|---|---|
| | | | Lys | Cyt | Rnase | Chy |
| 6.2 | POPC-coated | 60 | 14987 | 168710 | 498130 | 238538 |
| 6.2 | POPC-coated | 0[a] | 125616 | 124714 | 256942 | 138534 |
| 5.2 | POPC-coated | 60 | 13048 | 194628 | 267783 | 212742 |
| 5.2 | POPC-coated | 0[a] | 76000 | 76881 | 45338 | 37073 |
| 6.0 | Not dynamically coated | 120 | 214008 | 77277 | 180477 | 150000 |
| 5.0 | Not dynamically coated | 120 | 273538 | 254755 | 288591 | 204363 |
| 4.0 | Not dynamically coated | 120 | 226040 | 304567 | 378640 | 200946 |

Lys: lysozyme; Cyt: cytochrome C; Rnase: ribonuclease A; Chy: α-chymotrypsinogen A.
[a] To indicate capillary filled with POPC-free BGE.

Repeated runs of the basic proteins mixture were carried out to evaluate the repeatability of migration times by the two different approaches. The relative standard deviation (R.S.D.) of protein migration times ranged from 2.20 to 3.60% in absence of POPC liposomes in the BGE filled into the capillary (capillary dynamically coated) and from 2.90 to 4.40% in presence of the liposomes (capillary dynamically coated and filled with POPC liposome dispersed in the BGE).

*Experiments at pH 5.2*.

Due to the poor buffering capacity of phosphate at pH values ranging from 6.0 to 4.0, the experiments within this pH range were conducted using citrate buffer. Therefore,

experiments at pH 5.2 were performed with 20 mM citrate buffer, containing 68 mM NaCl (BGE) using the two strategies described in the experimental section (section 3.4). After the dynamic coating the EOF generated in the capillary was $7.97 \times 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ (S.D. $1.6 \times 10^{-10}$ $m^2$ $V^{-1}$ $s^{-1}$; R.S.D., 2.0%), which is about one fourth of the EOF measured before rinsing the capillary with the BGE containing the dispersed liposomes ($2.93 \times 10^{-8}$ $m^2$ $V^{-1}$ $s^{-1}$; S.D. $7.99 \times 10^{-10}$ $m^2$ $V^{-1}$ $s^{-1}$; R.S.D., 2.7%). The electrophoretic runs of basic proteins carried out with the POPC liposomes dynamically coated capillary in the presence (panel A) and in the absence (panel B) of liposomes in the BGE filled into the capillary are depicted by the electropherograms reported in Figure 12.

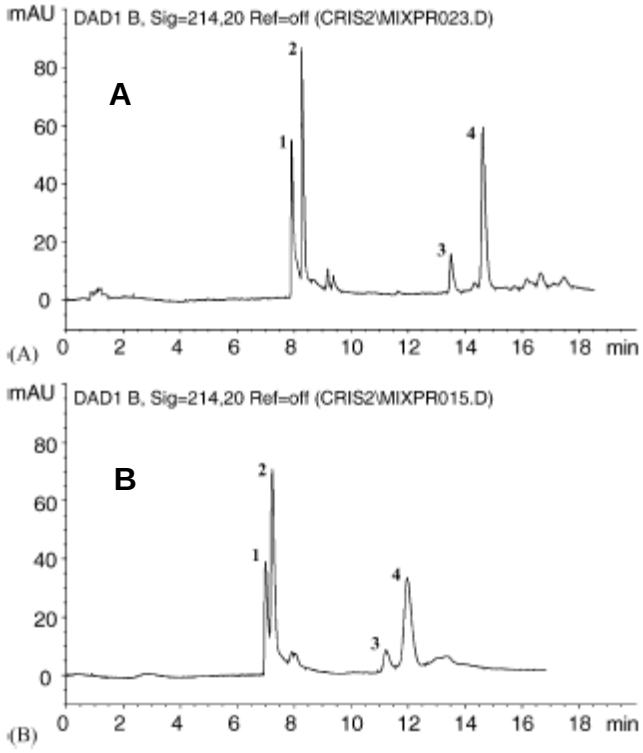



**Figure 12.** Electrophoretic behaviour of basic proteins at pH 5.2 in POPC dynamically coated capillary either filled with 60 μM POPC liposomes dispersed in the BGE (A) or with BGE (POPC-free BGE) (B). Electrolyte solution (BGE), 20mM citrate buffer pH 5.2, containing 68mM NaCl. Proteins, 1=lysozyme, 2=cytochrome c 3=ribonuclease A, 4=α-chymotrypsinogen A. Applied voltage, 10.0 kV; cathodic detection at 214 nm.

The electropherograms and data reported in Figure 12 and Tables 2 and 3 respectively, show that resolution and selectivity of the completely separated proteins were improved by the presence of POPC liposomes plug in the dynamically coated capillary (panel A), similarly to the behaviour already observed with phosphate buffer at pH 6.2 (Figure 11,

panel A). From this it can be inferred that the action of POPC liposomes in suppressing the adsorption of proteins onto the capillary wall may also result from the competitive interactions of proteins with the liposomes dispersed in the solution, rather than simply from the masking effect of POPC liposomes for the silanol groups on the capillary wall, which is the mechanism generally accepted for the additives adsorbed at the interface between the capillary wall and the electrolyte solution.[18]

*Experiments at pH 6.0-4.0 at constant ionic strength*.

Further experiments were performed in order to evaluate the influence of POPC liposome dispersed in the BGE filled into the capillary on the electrophoretic behaviour of basic proteins using bare fused-silica capillaries, which were not preventively treated for the dynamic coating with POPC liposomes. The experiments were carried out by injecting the basic proteins mixture after having completely filled the capillary with the different BGE containing POPC liposomes at various concentrations ranging from 30 to 120 μM by injecting into the capillary a plug of each solution equivalent to the volume of the capillary tube. Between runs the capillary was flushed with the BGE for 1.0 min with pressure of 90.0 kPa to remove the plug introduced before the previous sample injection. The study was performed using citrate buffer at three different pH values ranging from 6.0 to 4.0 and constant ionic strength of 0.111 M. Ionic strength was maintained at a constant value in order to level off the contribution of this parameter to the generation of the electric double layer at the interface between the capillary wall and the electrolyte solution and between the surface of liposomes and the electrolyte solution with changing pH.[19] Ionic strength is known to influence both the size of ion atmosphere around ions and colloidal particles and the thickness of the electric double layer at a solid-liquid interface.[20] In addition, ionic strength has proven to alter the size dependent electrophoretic behaviour of liposomes.[21] The electrophoretic behaviour exhibited by the basic proteins at pH 6.0, 5.0, and 4.0 in presence of POPC liposome dispersion, introduced into the capillary just before injecting the proteins sample, are depicted by the electropherograms displayed in Figure 13, whereas data on efficiency and resolution are reported in Tables 2 and 3. The concentration of POPC liposomes in

[18] (a) Doherty, E.A.S.; Meagher, R.J.; Albarghouthi, M.N.; Barron, A.E. *Electrophoresis* **2003**, *24*, 34-54. (b) Righetti, P.G.; Gelfi, C.; Verzola, B.; Castelletti, L. *Electrophoresis* **2001**, *22*, 603-611.
[19] Hiemenz, P.C. *Principles of Colloid and Surface Chemistry*, 2nd ed., Marcel Dekker, New York, **1986**.
[20] Adamson, A.W. *Physical Chemistry of Surface*, 5th ed., Wiley Interscience, New York, **1990**.
[21] Physer, M.D.; Hayes, M.A. *Langmuir*, **2004**, *20*, 4369-4375.

the BGE filled into the capillary was 120 μM. This concentration was selected on the basis of the results obtained at pH 6.0 using solutions containing POPC liposomes at concentration lower than 120 μM, which were more unsatisfactory than those displayed by the top chromatogram of Figure 13 due to the poor resolution of ribonuclease A from α-chymotrypsinogen A observed at this pH value with citrate buffer.

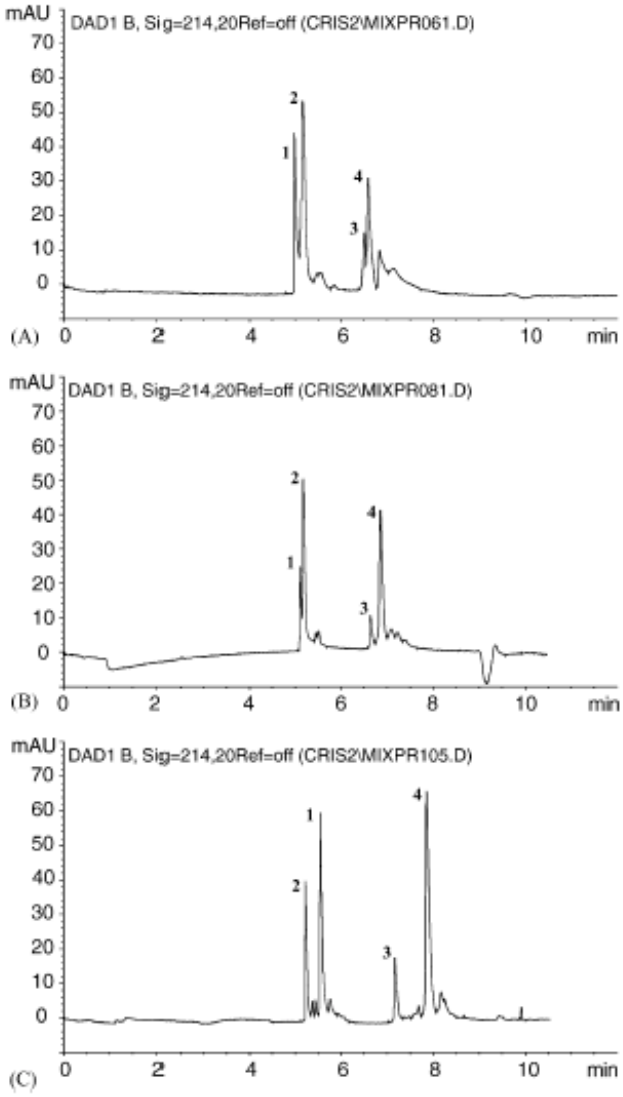



**Figure 13.** Electrophoretic behaviour of basic proteins in bare fused-silica capillary filled with one column volume of 120 μM POPC liposomes dispersed in BGE of constant value of ionic strength (0.111 M) and different pH values. (A): pH 6.0; (B): pH 5.0; (C): pH 4.0. Electrolyte solution (BGE), 20mM citrate buffer of constant value of ionic strength (0.111 M) at pH 6.0 (A), pH 5.0 (B), and pH 4.0 (C). Proteins, 1=lysozyme, 2=cytochrome c 3=ribonuclease A, 4=α-chymotrypsinogen A. Applied voltage, 10.0 kV cathodic detection at 214 nm.

With lower concentrations of POPC liposomes all proteins exhibited higher mobility and lower resolution (see below). The significant different migration behaviour of the

protein pair α-chymotrypsinogen A and ribonucleaseA observed with pH 6.2 phosphate and with pH 6.0 citrate buffers (Figures 11 and 13) can be related to the selective ion-pair formation of these basic proteins with phosphate and citrate anions. Such effect is expected to vary the effective charge-to-hydrodynamic radius of proteins and hence their electrophoretic mobility, as it has already described to elucidate the influence of buffer anions on the electrophoretic mobility of proteins.[8c] However, it should be recall that in absence of POPC liposomes all basic proteins were irreversibly adsorbed on the capillary wall of the bare fused-silica capillary. Therefore, it can be inferred that POPC liposomes influence significantly the electrophoretic behaviour of basic proteins, either in the absence or in the presence of the preventive dynamic coating of the capillary wall with the liposomes. The dependence of the electrophoretic behaviour of basic proteins from the concentration of POPC liposomes dispersed in the BGE filled into the capillary before injecting the proteins sample investigated at pH 4.0 with the concentration of POPC liposomes in the BGE filled into the capillary ranging from 30 to 120 μM in 20 mM citrate buffer at pH 4.0 and constant ionic strength (0.111 M) is depicted in Figure 14 as plots of the migration times against the concentration of dispersed POPC liposomes.

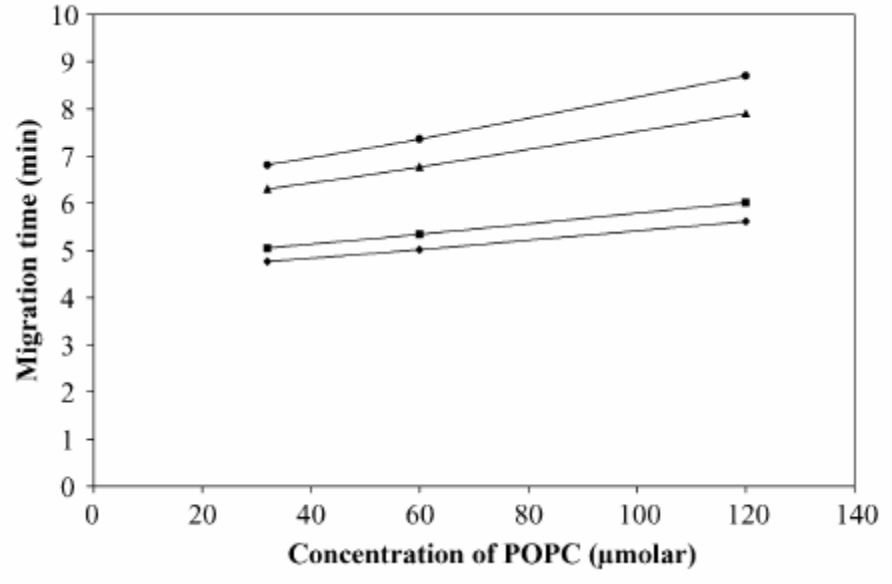



**Figure 14.** Plots of migration times as a function of POPC liposomes concentration in 20mM citrate buffer pH 4.0 for the four basic proteins: (♦) cytochrome *c*, (■) lysozyme, (▲) ribonuclease A, (●) α-chymotrypsinogen A. Cathodic detection at 214 nm.

It is noted that the migration times of all four proteins increases with increasing the concentration of POPC liposomes in the BGE introduced into the capillary before injecting the proteins sample, indicating significant interactions between proteins and POPC liposomes. This is further evidenced in Figure 15, where the intrinsic selectivity of the system, defined as the ratio of the mobility of faster moving analyte to that of the slower one,[22] is plotted as a function of the concentration of POPC liposomes for the two pairs proteins cytochrome *c*/lysozyme and α-chymotrypsinogen A/ribonuclease A.

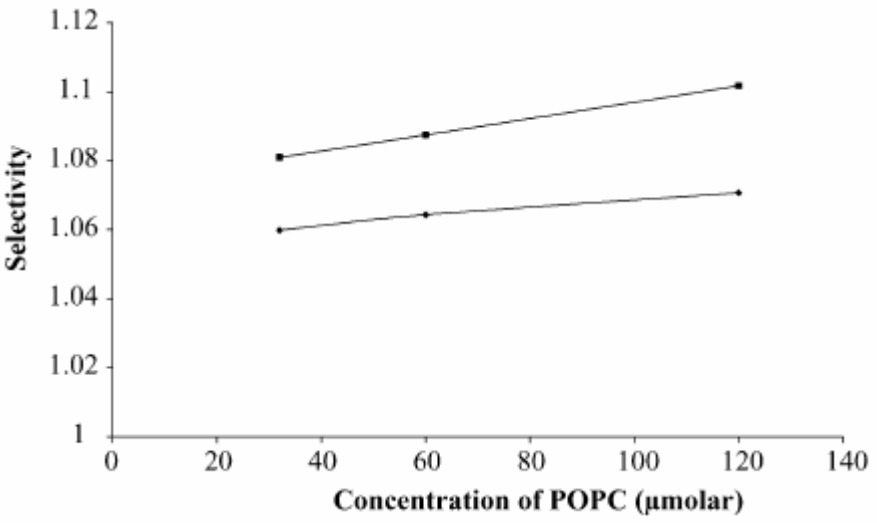



**Figure 15.** Plots of the selectivity for the two protein pairs: (♦) cytochrome *c* and lysozyme; (■) ribonuclease A and α-chymotrypsinogen A as a function of POPC liposomes concentration in 20mM citrate buffer pH 4.0. Cathodic detection at 214 nm.

For both protein pairs the selectivity increases almost linearly, but with different slope, with the concentration of POPC liposomes present in the capillary. The above findings suggest that the variations in selectivity should be the results of different interactions of proteins with POPC liposomes rather than the effect of the variations in parameters such as the electro-osmotic flow or the viscosity of the liposome dispersion, that are expected to contribute indifferently to the mobility of samples. On the other hand, it should be considered that filling a bare fused-silica capillary with POPC liposomes dispersed in the BGE has the effect of suppressing the interaction of basic proteins with the silanol groups on the capillary wall. However, this effect could only explain the effectiveness of POPC liposomes at preventing protein–capillary wall interaction without justifying the differences in selectivity observed for the two protein pairs with increasing POPC liposomes concentration. It is worth mentioning that at pH values higher than 4.0 the

[22] Rathore, A.S.; Horvath, Cs. *J. Chromatogr. A*, **1996**, *743*, 231-.

migration order of the protein pair lysozyme–cytochrome *c* is reversed with respect to that observed at pH 4.0. This is the results of the increase of the electrophoretic mobility with decreasing pH, which below pH 5.0 is greater for cytochrome *c* than for lysozyme. The effect has been evidenced with a variety of electrolyte solutions by plotting the electrophoretic mobility of the two proteins as a function of pH.[8a,8c] As expected, such plots show for both basic proteins the increasing of electrophoretic mobility with decreasing pH (in accordance with the increasing degree of protonation of amino groups) and the inversion of migration order of cytochrome *c* and lysozyme arising from the different slopes of the curves describing the dependence of the electrophoretic mobility from pH for these proteins, which cross each other between pH 4.0 and 5.0.

### *3.3 Circular Dichroism*.

In the previous paragraph the crucial role of the surface electrostatic interactions between the positively charged proteins and negatively charged POPC liposomes in the electrophoretic behaviour of proteins has been demonstrated. In order to further investigate the type of interactions between POPC liposomes and basic proteins, circular dichroism experiments were carried out. Circular Dichroism, CD, is a powerful technique that concerns the interactions between circularly polarized light and optically active molecules and provides structural information such as conformational changes of biopolymers (i.e. DNA or proteins) in biological systems. In particular CD spectroscopy has been extensively used in the structural characterization of peptides for the monitoring of conformational changes and for the estimation of the helical content.[23]

Secondary structure of the proteins can be determined by CD spectroscopy in the UV spectral region between 190 and 250 nm. At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in chiral regular, folded environment. Each Alpha-helix, beta-sheet, and random coil structures give rise to a characteristic shape and magnitude of CD spectrum. This is illustrated in Figure 16, which shows spectra for poly-lysine in these three different conformations.

[23] Rodger, A.; Norden, B. *Circular Dichroism and Linear Dichroism*, **1997**, Oxford University Press, New York.

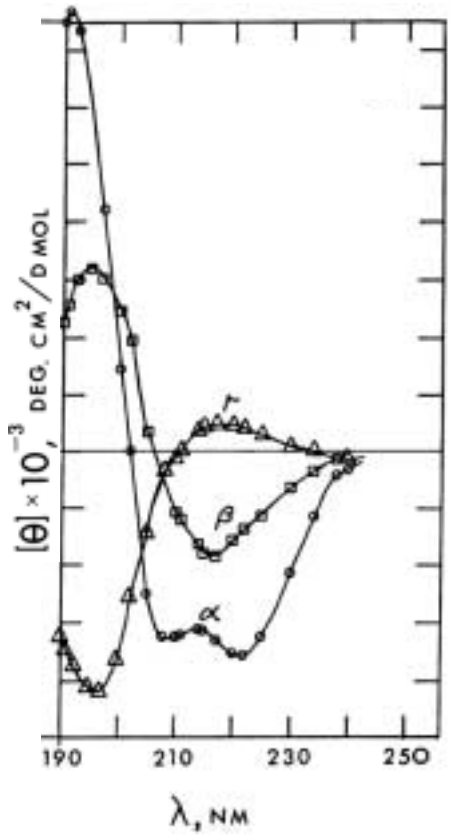



**Figure 16.** Circular dichroism spectra of poly-lysine in three different conformations: alpha-helix (α), beta-sheet (β) and random coil (r).

The approximate fraction of each secondary structure type that is present in any protein can thus be determined by analyzing its CD spectrum as a sum of fractional multiples of such reference spectra for each structural type.

Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population. Thus, while CD can assess that a protein contains about 50% alpha-helix, it cannot assess which specific residues are involved in the alpha-helical portion.

Concerning the CD experiments performed with POPC liposomes and basic proteins, the protein/liposome molar ratio (about 1:30 for each protein) was selected on the basis of the protein/ liposome molar ratio injected into the capillary tube.

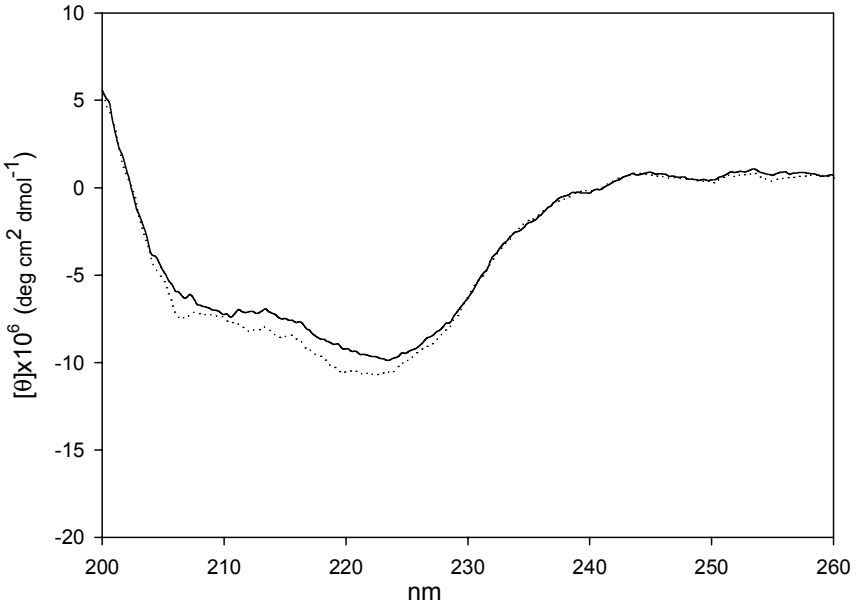



**Figure 17.** Circular dichroism spectra of cytochrome *c* in the absence (solid line) and in the presence of POPC liposome dispersion. cyt / POPC molar ratio, 1:30 (dotted line). Experiments carried out in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl.

Figure 17 shows the CD spectrum of cytochrome *c* in the presence (dotted line) and in the absence (solid line) of POPC liposomes dispersion in 20 mM phosphate buffer pH 7.4, containing 68 mM NaCl. It can be observed that the secondary structure of the cytochrome *c* (mainly composed of alpha-helix)[24] has not been substantially changed upon the addition of POPC liposome dispersion; this evidence suggests that the protein is not embedded in the hydrophobic portion of the liposome; the inclusion in the hydrophobic region would, in fact, affect its secondary structure.

[24] The content of alpha-helix, beta-sheet and random coil of the basic proteins were calculated by Protein Data Bank in the following PDB files: 2LYZ (lys); 1AKK (cyt); 1C0B (Rnase); 1CHG (Chy) available at www.rcsb.org.

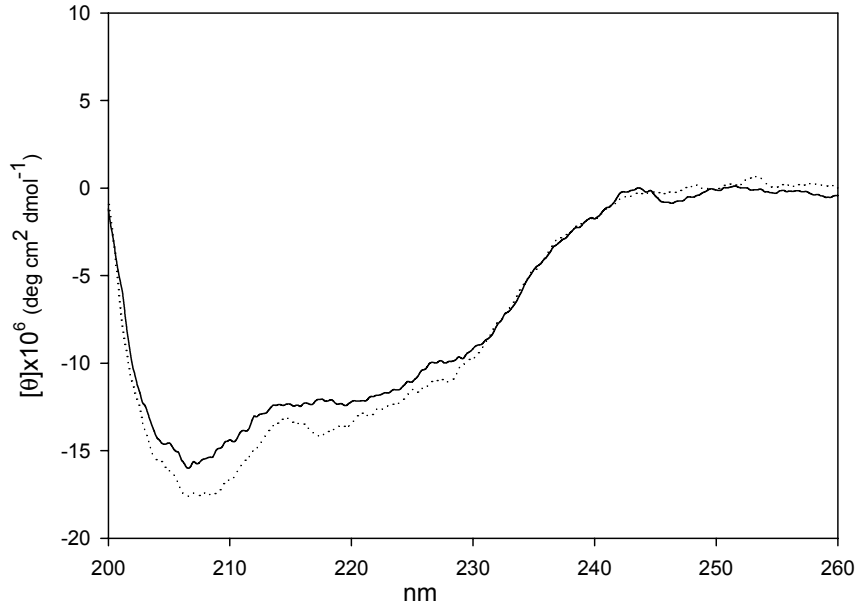



**Figure 18.** Circular dichroism spectra of lysozyme in the absence (solid line) and in the presence of POPC liposome dispersion. lys / POPC molar ratio, 1:30 (dotted line). Experiments carried out in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl.

Figure 18 shows the CD spectrum of lysozyme in the presence (dotted line) and in the absence (solid line) of POPC liposome dispersion in 20 mM phosphate buffer pH 7.4, containing 68 mM NaCl. Also in the case of lysozyme, the secondary structure (mainly composed of alpha-helix) has not been affected by the presence of POPC liposomes. The experiments performed on ribonuclease A and α-chymotrypsinogen A (mainly composed of random coil structure) gave similar results confirming the major role of electrostatics in the association of hydrophilic proteins with POPC liposomes.

### *3.4 Conclusions*

These studies have evidenced the potentiality of capillary electrophoresis for characterising phospholipid liposomes, for investigating biopolymers-liposomes interactions and for separating closely related peptides and basic proteins. Characterization of POPC liposomes by CE showed a consistent Gaussian shape, indicating homogeneous and relatively narrow size distribution of the liposomes in accordance with the dynamic light scattering measurements.

The POPC liposomes show strong affinity for the capillary wall of bare-fused silica capillaries, resulting in the variation of the electro-osmotic flow which depends both from the volume and from the concentration of the POPC liposomes dispersion injected into the capillary. Neverthless at pH 7.4 the dynamic coating of the capillary wall is not stable and could be removed by simply rinsing the capillary tube with the BGE.

The interactions of peptides and proteins with POPC liposomes were investigated at differents pH and composition of the BGE. The peptide/protein-liposome interactions have been revealed by the difference on the migration times, resolution and selectivity with respect to the absence of the liposome dispersion introduced into the capillary. In particular, the occurrence of selective interactions between peptides or proteins and liposomes can be inferred from the different variations in the relative mobility of the peptide or protein pairs. The main mechanism involved in the association of peptides/proteins with the phospholipids containing liposomes appears primarily an electrostatic interaction between the negatively charged polar headgroups of the liposomes, though also additional hydrophobic interactions appear to be involved. The different interactions between three related peptides and phospholipid liposomes should be due to the presence of positively or negatively charged and hydrophobic (at physiological pH) amino acidic residues that could differently interact with the negatively charged outer surface and the hydrophobic environment of POPC liposomes.

Concerning the basic protein-liposome interactions, it should be considered that filling a bare fused-silica capillary with POPC liposomes dispersed in the BGE has the effect of suppressing the interaction of basic proteins with the silanol groups on the capillary wall. However, this effect could only explain the effectiveness of POPC liposomes at preventing protein–capillary wall interaction without justifying the differences in selectivity observed for the four basic proteins with increasing POPC liposome concentration. The competitive interactions may account for the efficient separation of basic proteins obtained in a bare-fused silica capillaries in all the conditions.

Circular dichroism measurements performed in order to better understand the nature of the interactions of basic proteins with the lipidic double layer showed that the secondary structure of the proteins has not been substantially changed by the addition of the POPC liposome dispersion. From this result it can be inferred that the proteins are not

embedded in the hydrophobic portion of the liposome. These findings are in full agreement with the hypothesis formulated on the basis of CE results.

## *3.5 Experimental Section*

### *3.5.1 Preparation of Phospholipid Liposomes*

Liposomes consisting of large unilamellar vesicles (LUV) were prepared by modifying extrusion technique reported in literature,[25] as described below. The desired amount of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was dissolved in chloroform in a round-bottomed flask. The round-bottomed flask was placed in a rotary evaporator with a water bath at 30-35°C to produce a thin layer of phospholipids on the inner surface of the flask. The residue traces of the solvent were removed under high vacuum overnight. The resulting thin lipid film was hydrated, under shaking, with 10 mM phosphate buffer at pH 7.4, containing 2.7 mM potassium chloride and 137 mM sodium chloride, to give a dispersion of multilamellar vesicles (MLV) with a lipid concentration of 40 mM. The MLV dispersion was subjected to five freeze-thaw cycles[26] from liquid nitrogen in order to reduce multilamellarity and processed to LUV by extrusion[27] through a polycarbonate filters of 0.2 and 0.1 μm pore size. First, the dispersion was passed ten times through a 0.2 μm pore size polycarbonate filter (Whatman, Maidstone, UK) and then 10 times through a 0.1 μm pore size. The extrusion were carried out above the main transition temperature of POPC (4°C) using a 2.5 mL Lipex Model T001 extruder (Lipex Biomembrane Vancouver, Canada). The prepared liposomes dispersion was stored at 4°C in the dark.

### *3.5.2 Capillary Electrophoresis*

All the experiments were performed using an HP$^{3D}$ Capillary Electrophoresis system from Agilent (Waldbronn, Germany), consisting of a high voltage power supply, a diode array UV–vis detector and an air-cooling device for temperature control of the cartridge containing the capillary tube. The capillary electrophoresis unit was interfaced with an HP Vectra XM 5 166MHz personal computer running the HP$^{3D}$CE ChemStation software, providing system control in addition to data acquisition and evaluation.

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[25] (a) Hope, M.J.; Bally, M.B.; Webb, G.; Cullis, P.R. *Biochim. Biophys. Acta* **1985**, *812*, 55-65. (b) Hope, M.J.;Mayer, L.D.; Cullis, P.R. *Biochim. Biophys. Acta* **1986**, *858*, 161-168.

[26] Vemuri, S.; Rhodes, C.T. *Pharm. Acta Helv*. **1995**, *70*, 95-111.

[27] Hope, M.J.; Nayar, R.; Mayer, L.D.; Cullis, P.R. Reduction of Liposome Size and Preparation of Unilamellar Vesicles by Extrusion Techniques in *Liposome Technology* 2nd Ed.; G. Gregoriadis ed., CRC Press: Boca Raton, Florida, **1992**; Vol. 1, chapter. 8.

Capillary tube of 0.050mm I.D. and 0.375mm O.D. was purchased from Quadrex (New Haven, CT, USA). Capillaries having total length of 330mm and distance from the detection window and capillary end of 85mm were prepared in house. Prior to use for the first time, the new fused-silica capillary was flushed successively with 0.5 M sodium hydroxide (30 min), water (10 min), and 0.5 M hydrochloric acid (30 min), followed by a second treatment with water (10 min), 0.5 M sodium hydroxide (30 min), water (10 min).[8c] All experiments were carried out at constant applied voltage of 10 kV with the temperature of the capillary cartridge set at 25°C. Samples were introduced into the capillary by pressure injection at 5 kPa for 3.0 sec. Before the plug of the liposomes dispersion or analytes injection, the capillary was preconditioned with appropriate buffer which was the background electrolyte solution (BGE) used in the capillary experiments. After preconditioning the capillary with the BGE, the electro-osmotic flow was measured on the basis of the migration time of the neutral marker mesityl oxide. The investigations about the interactions between liposomes and biopolymers were carried out by different approaches.

*Characterization of the phospholipid liposomes dispersion*

The capillary was first preconditioned with 20 mM phosphate buffer, pH 7.4 containing 68 mM NaCl. The liposome dispersion, containing 40 mM POPC, was diluted 5 times with the BGE and introduced into the capillary by pressure injection at 5 kPa for 3.0 s. Between runs, the capillary was consecutively rinsed with water (1.0 min), 1.0 M sodium hydroxide (1.0 min), water again (1.0 min) and then with the BGE for 5.0 min. After this treatment, the electro-osmotic flow was measured by injecting mesityl oxide. Based on the unchanged value of electro-osmotic flow, the capillary appeared to be reconditioned to the initial conditions before each run of POPC sample.

*Influence of liposomes on the electroosmotic flow*

The study was first performed by injecting POPC liposomes plugs of different volumes ranging from 50 to 520 nL and measuring the electro-osmotic flow after each plug injection, and then by rinsing the capillary with the POPC liposomes dispersion by different rinsing times ranging from 5 to 20 min, all exceeding the time requested for replacing one volume of the capillary column (250 s, 650 nL). The experiments were carried out with the dispersion containing 60 μM POPC liposome dispersion. The volume of plug of liposomes dispersion injected was just about calculated by the CE

Expert Version 1.0 software from Beckman (Fullerton, CA, USA) on the basis of the injection time and applied pressure with the approximation of having considered the viscosity of POPC liposome dispersion equal to that of distilled water, while capillary dimensions and temperature of the capillary cartridge requested by the software were those reported above. Concerning the investigation about the effect on the electro-osmotic flow liposome dispersion of different concentration of POPC liposome dispersion, the study was conducted by rinsing sequentially the capillary with a POPC liposome dispersion of increasing concentration of POPC, ranging from 30 to 200 μM.

*Liposome Capillary Electrophoresis of Peptides*

The studies about the interactions between liposomes composed of the zwitterionic 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) and biopolymers were conducted by evaluating different approaches of performing liposome capillary electrophoresis. A first strategy consisted in partially filling the capillary tube with a POPC liposome dispersion in the BGE. Plugs of different volume (calculated as reported above) were introduced into the capillary tube by injecting hydrodynamically at 5 kPa, POPC liposome dispersion at different concentrations. The complete capillary filling approach was realized by injecting a POPC liposomes plug of 1000 nL, corresponding to about 1.5 times the volume of the capillary tube. Between runs the capillary was flushed with the BGE to remove the plug introduced before the sample injection.

*Liposome Capillary Electrophoresis of Proteins*

Concerning the experiments performed at pH 7.4, the capillary tube was dynamically coated and filled with a 60 μM POPC liposomes dispersed in BGE solution which was also employed as running electrolyte solution. Between runs capillary was rinsed with 60 μM POPC liposome dispersed in BGE solution in order to refreshing the coating and filling again the capillary tube with the POPC liposome dispersion. In the investigation about the influence of pH and composition of BGE (phosphate and citrate buffers) on the interactions between liposomes and basic proteins, POPC liposomes were employed both for the coating of the capillary tube and as a pseudo stationary phase. In the dynamically coating approach, capillary tube was first rinsed for a given time period with pressure of 5.0 kPa with a 60 μM POPC liposomes dispersed in BGE solution and then rinsed with BGE not containing POPC liposomes (i.e. POPC-free BGE) in order to

remove the liposomes not adsorbed on the capillary wall and to have a dynamic coating on the inner wall of the capillary tube. POPC-free BGE was also employed as running electrolyte solution. Between runs capillary was rinsed with the 60 μM POPC liposomes dispersed in BGE for 5.0 min at 5 kPa in order to renew the coating followed by a 1.0 min at 90 kPa rinse of POPC-free BGE. In the pseudo stationary phase approach, the capillary was dynamically coated and filled with 60 μM POPC liposomes dispersed in BGE solution. Between runs the capillary was rinsed with 60 μM POPC liposomes dispersed in BGE solution for 1.0 min with pressure of 90 kPa in BGE solution in order to refresh the coating and to fill again the capillary tube with the POPC liposome dispersion.

### *3.5.3 Dynamic Light Scattering*

Dynamic light scattering (DLS) measurements, which were carried out using a Zetasizer 5000 (Malvern Instruments, Malvern, UK) consisting of a photomultiplier tube, a Malvern 7132 multi-bit digital correlator, and a 5mW He–Ne laser. All experiments were performed at the scattering angles of 90° ($\lambda$ = 488 nm) and thermostatically controlled at 25°C. Hydrodynamic diameter of liposomes was determined from the cumulant analysis of the intensity autocorrelation function.[28] The vesicle size was taken as a mean value of three replication of 10 measurements. The particle size distribution (PSD), computed by an inverse Laplace transformation of the data, were analyzed with the algorithms NNLS (non-negative least squares, smoothing 0.005) and CONTIN built-in procedures of the Malvern Zetasizer 5000. Original POPC dispersion of 40 mM was diluted 100 times.

### *3.5.4 Circular Dichroism*

CD measurements were carried out with a Jasco spectropolarimeter J-715 (Jasco, Easton, MD). Spectra were acquired in a 0.5-cm path length quartz cuvette and were measured as the average of three scans from 200 to 350 nm at a scan rate of 50 nm/min. CD spectra were registered at $T$ = 298 K immediately after the addition of the liposome dispersion to the proteins.

[28] Cheng, Z.; Luisi, P.L. *J. Phys. Chem*. **2003**, *107*, 10940-.

### *3.5.5 Chemicals and Samples*

Reagent-grade phosphoric acid, citric acid, sodium hydroxide, sodium chloride, and chloroform were purchased from Carlo Erba (Milan, Italy). Deionized water was obtained by a Milli-Q water purification system from Millipore (Bedford, MA, USA) and degassed by sonication before use. Mesityl Oxide was obtained by Fluka (Milan, Italy). Cytochrome c (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas) and α-chymotrypsinogen A (from bovine pancreas), synthetic peptides having the amino acid sequence of human angiotensin I (DRVYIHPFHL), angiotensin II (DRVYIHPF), and angiotensin III (RVYIHPF) were obtained from Sigma (Milan, Italy), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

### *3.5.6 Buffers*

Buffers consisting of either a monoprotic or a polyprotic acid and its sodium salt were prepared by titrating the proper amount of requested acid to the desired pH value with 1.0 M sodium hydroxide. Buffers of constant ionic strength were prepared in a similar way with the incorporation of the proper amount of sodium chloride calculated as described in literature.[29] The pH was measured with a glass electrode Model 52–02 and Model Basic 20 pH-Meter, both from Crison (Alella, Spain). All solutions were filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sonication before use.

[29] Perrin, D.D.; Dempsey, B. *Buffers for pH and Metal Ion control*, Chapman & Hall, London, **1974**.

# Chapter 4- *Cationic Liposome Capillary Electrophoresis of Basic Proteins*

## *4.1 Introduction*

Cationic liposomes used in our investigation are composed of binary mixtures of natural phospholipids and cationic gemini surfactant. Nature of cationic additives, can strongly influence the properties of the cationic liposomes. Among cationic additives, a unique class is represented by cationic gemini surfactants; gemini surfactants are characterized by two polar headgroups, connected by a spacer of various kinds, and two hydrophobic tails. This structural feature provides gemini unique properties among the surfactants; they, for example, form aggregates at a concentration almost a hundredfold lower than the corresponding single chain surfactants and even have surface activities thousandfold higher[1] and represent an interesting field both for industry[2] and academic research.[1,3]

For the preparation of cationic liposomes, practically all standard methods of liposome preparation are applicable (as described in chapter 1, section 1.4). Due to very high charge densities, cationic liposomes disperse better than neutral ones and in some cases it could be formed small liposomes by vigorous vortexing of the hydrating lipid films.[4]

Cationic liposomes can be characterize by identical method used for neutral and anionic ones (as described in chapter 1, section 1.4).

In a recent application the use of a cationic gemini surfactant mixed with natural phospholipids showed an improvement of the interactions of the lipidic double layer with the phosphate groups present in DNA and a better inclusion of a photosensitizer in the lipidic double layer[5] with respect to the use of an analogous single chain cationic surfactant.

This chapter reports and discusses the results of a study carried out to investigate the interactions between proteins and mixed liposomes formed by phospholipids and a cationic gemini surfactant. Aim of this work is exploring the suitability of liposome capillary electrophoresis for investigating how the modification of the liposome surface

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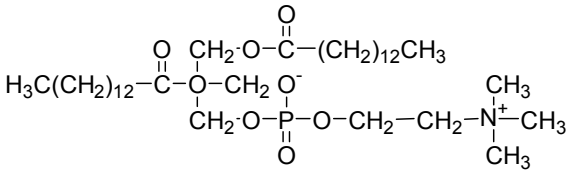
[1] Menger, F.M.; Keiper, J.S. *Angew. Chem. Int. Ed*. **2000**, *39*, 1906-1920.
[2] (a) Kwetkat, K. WO 9731890, **1997**. (b) Dahms, G.; Kwetkat, K. DE 19943681, **2001**.
[3] (a) Perez, L.; Torres, J.L.; Manrese, A.; Solans, C.; Infante, M.R. *Langmuir*, **1996**, *12*, 5296-5301. (b) Menger, F.M.; Mbadugha, N.A. *J. Am. Chem. Soc.* **2001**, *123*, 875-885.
[4] Lasic, D.; Templeton, N.S. *Advanced Drug Deliv. Rev*. **1996,** *20,* 221-226.
[5] (a) Bombelli, C.; Caracciolo, G.; Di Profio, P.; Diociaiuti, M.; Luciani, L.; Mancini, G.; Mazzuca, C.; Marra, M.; Molinari, A.; Monti, D.; Toccaceli, L.; Venanzi, M. *J. Med. Chem*. **2005**, *48*, 4882-4891. (b) Bombelli, C.; Fagioli, F.; Luciani, P.; Mancini, G.; Sacco, M.G. *J. Med. Chem*. **2005**, 48, 5378-5382.

(i.e. cationic liposomes) could influence their interactions with proteins. Moreover the capacity of cationic liposome capillary electrophoresis for separating analytes which display strongly interactions with the inner capillary wall of the bare-fused silica capillaries, was investigated. The investigation was performed with unilamellar cationic liposomes extruded at 100 nm.

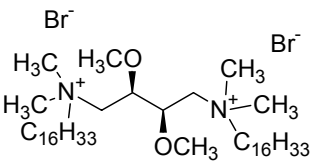
## *4.2 Results and Discussion*

### *4.2.1 Influence of Liposomes on the Electro-osmotic Flow*

Because positively charged cationic liposomes are expected to strongly interact with the negatively charged inner surface of bare fused-silica capillaries at pH 7.4 with the result of influencing the electro-osmotic flow, we investigated the effect on the electro-osmotic flow of cationic liposomes composed of the phospholipid 1,2-dimiristoyl-sn-glycero-3-phosphocholine (DMPC) and the cationic gemini surfactant (2S,3S)-2,3-dimethoxy-1,4-bis(*N*,*N*-dimethyl-*N*,-hexadecylammonium) butane dibromide (SS) in a molar ratio 9:1 dispersed in 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl. The cationic liposomes were introduced into the capillary as plugs of different volumes.



DMPC



SS

The study was performed by introducing the DMPC/SS 9:1 liposome dispersion as plugs of different volumes into a bare fused-silica capillary and measuring the electro-osmotic flow after each plug injection. The values of the coefficient of the electro-osmotic flow plotted as a function of the volume of the liposome dispersion containing 60 μM of DMPC/SS 9:1 are reported in Figure 1.

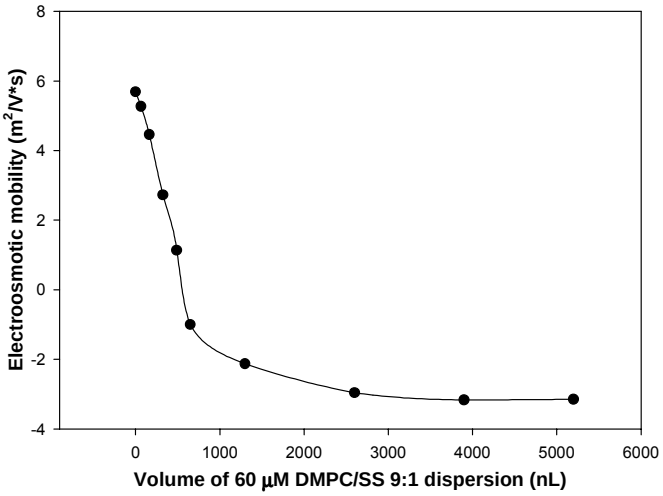



**Figure 1.** Effect of plug volume of a 60 µM DMPC/SS 9:1 liposomes dispersed in the BGE on the electro-osmotic flow. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl; applied voltage, ±10.0 kV; cathodic/anodic detection at 214 nm.

From the plot of the coefficient of the electro-osmotic flow as function of the volume of DMPC/SS 9:1 liposome dispersion introduced into the capillary, it can be observed that increasing the volume of the plug introduced into the capillary has the effect of gradual decreasing and subsequent reversing of the direction of the EOF from cathodic to anodic. Rinsing the capillary tube with a volume of the 60 µM DMPC/SS 9:1 liposome dispersion corresponding to 3/4 of the volume of the capillary tube (490 nL), resulted in decreasing the electro-osmotic flow down to $1.13 \times 10^{-8}$ $m^2V^{-1}s^{-1}$, which is about four times lower than the value measured in absence of the cationic liposome dispersion ($5.69 \times 10^{-8}$ $m^2V^{-1}s^{-1}$). When the capillary was completely filled with a plug of DMPC/SS 9:1 liposome dispersion corresponding to the volume of the capillary tube (650 nL), the direction of the electro-osmotic flow was reversed from cathodic to anodic and the measured value of the coefficient of the electro-osmotic flow was $-1.00 \times 10^{-8}$ $m^2V^{-1}s^{-1}$, the negative sign indicating the anodic direction of EOF.

Flushing the capillary tube with a volume of the cationic liposome dispersion corresponding to about nine times the volume of the capillary tube (5.2 µL) resulted in increasing the anodic electro-osmotic flow to a maximum value ($-3.15 \times 10^{-8}$ $m^2V^{-1}s^{-1}$) that practically did not varied by flushing the capillary with larger volumes of the liposome dispersion. The reversal of the direction of the electro-osmotic flow indicates the inversion of the zeta-potential at the plane of share between the Stern and mobile region of the electric double layer (Figure 2).

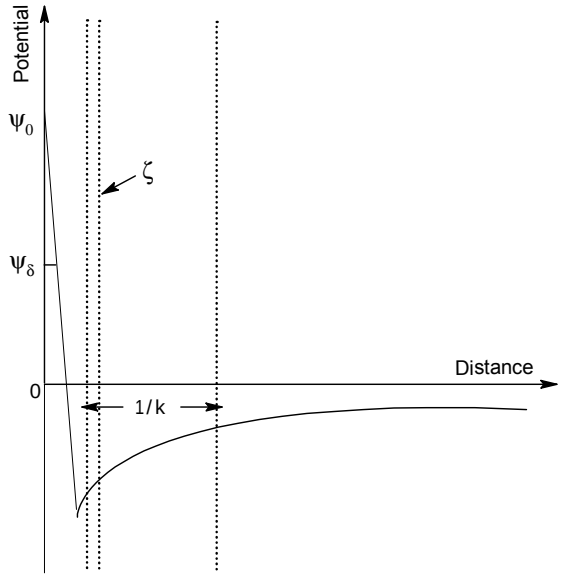



**Figure 2.** Schematic representation of the electric double layer concerning the adsorption of cationic liposomes at a solid-liquid interface and variation of potential with the distance from the solid surface: $\psi_o$, surface potential; $\psi_\delta$, potential at the Stern plane; $\xi$, potential at the plane of share (zeta potential); $\delta$, distance of the Stern plane from the surface (thickness of the Stern layer); $k^{-1}$, thickness of the diffuse region of the double layer.

The reversal of the direction of the electro-osmotic flow from cathodic to anodic evidences the specific adsorption of the liposomes at the solid-liquid interface with consequent variation of the charge density in the Stern region of the electric double layer (section 2.1).[6] In such a case the cationic liposomes are believed to be firmly held in the compact region of the electric double layer by additional forces besides those of simple coulombic origin. The specific adsorption of liposomes at the interface between the capillary wall and the electrolyte solution results in a drastic variation of the positive charge density in the Stern layer, which reduces the zeta potential and, hence the electro-osmotic flow, as it is observed with the partial filling of the capillary tube with the liposome dispersion. When the positive charge density of the adsorbed counter-ions exceeds the negative charge density on the capillary wall, resulting from the ionization of silanol groups, the zeta potential becomes positive and the electro-osmotic flow is reversed from cathodic to anodic, as it is observed upon filling the capillary with the cationic liposome dispersion. Rinsing the capillary for 20 minutes at 90 kPa with BGE caused the reversal of the EOF from anodic to cathodic ($0.23 \times 10^{-8}$ $m^2V^{-1}s^{-1}$). It was

[6] Corradini, D. *Electro-Osmotic Flow* in Encyclopedia of Chromatography, **2001**, Jack Cazes ed., Marcel Dekker, New York.

necessary to flush the capillary tube with sodium hydroxide 1.0 M to restore the electro-osmotic flow to its original values determined in absence of DMPC/SS 9:1 liposome dispersion into the capillary tube. These findings indicate that the dynamic coating of the capillary tube obtained with the cationic liposome dispersion is relatively stable and can be completely removed only treating the inner surface of the capillary with a strong alkaline solution.

### *4.2.2 Cationic Liposome Capillary Electrophoresis of Basic Proteins*

The study on the interactions between cationic liposomes (CL) and biopolymers was performed examining the electrophoretic behaviour of four water soluble basic proteins lysozyme, cytochrome C, ribonuclease A and α-chymotrypsinogen A (see section 3.2.5). A first set of experiments was performed at pH 7.4 in a dynamically coated capillary with a cationic liposome composed of the phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and the cationic gemini surfactant (2S,3S)-2,3-dimethoxy-1,4-bis(*N*,*N*-dimethyl-*N*,-hexadecylamonium) butane dibromide (SS) in a molar ratio 9:1.

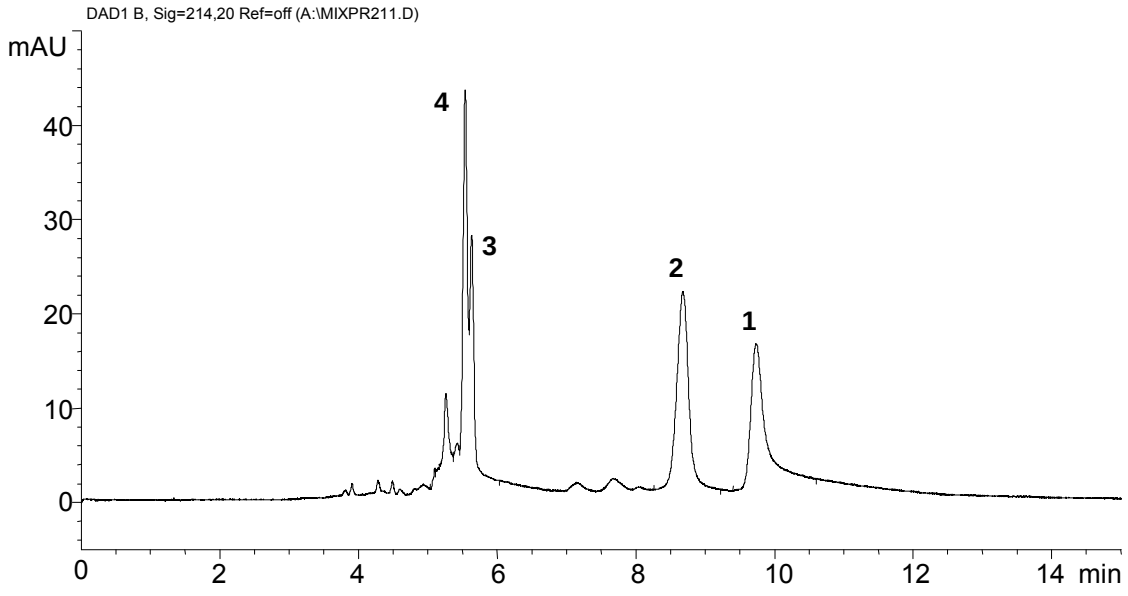



**Figure 3.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated with 60 μM DMPC/SS 9:1 liposomes dispersed in BGE. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

Being the electro-osmotic flow reversed, the migration order of the basic proteins (Figure 3) was reversed with respect to the migration order observed in liposome phospholipid capillary electrophoresis of basic proteins at pH 7.4 (as described in section 3.2.5). At pH 7.4 both basic proteins and CL composed of DMPC/SS 9:1 are positively charged and the electrostatic interactions betweens liposomes and proteins are of repulsive type. It can be noticed that ribonuclease A and α-chymotrypsinogen A are not completely resolved, in accordance to the very close values of the electrophoretic mobility of these proteins at pH 7.4 in electrolyte solutions containing phosphate ions.[7]

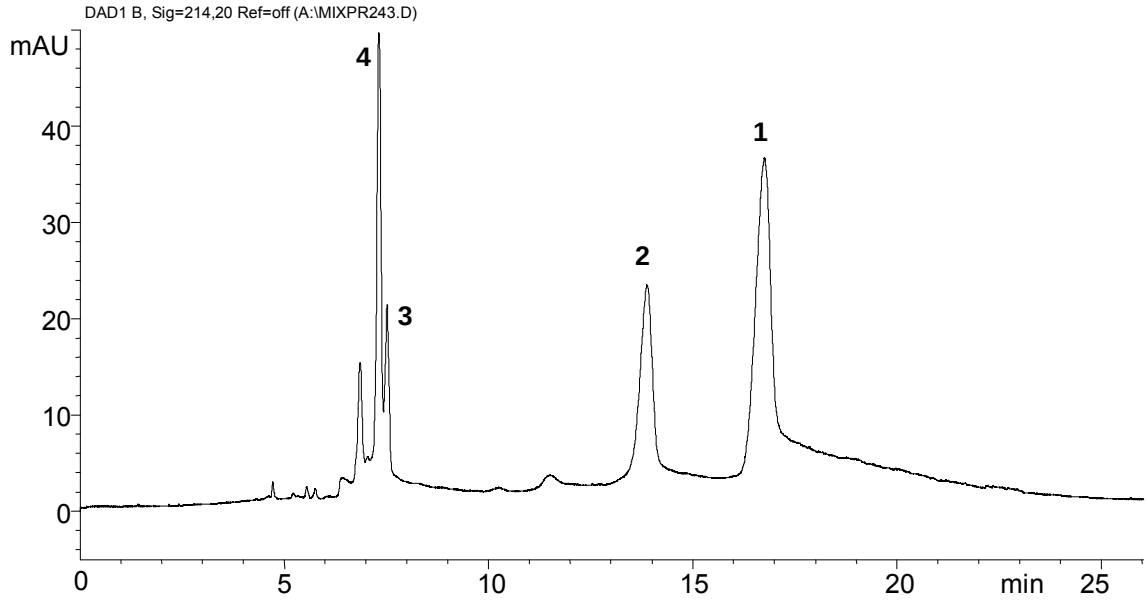



**Figure 4.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated with 60 μM POPC/SS 9:1 liposomes dispersed in BGE. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

Changing the phospholipid counterpart, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; section 3.2.1) instead of DMPC in the same molar ratio (9:1), did not produce substantial differences on the electrophoretic behaviour of the basic proteins (Figure 4), although the apparent mobility of all proteins was lower than the apparent mobility observed with the DMPC/SS liposomes coated capillary, due to the lower electro-osmotic mobility ($-2.73 \times 10^{-8}$ $m^2V^{-1}s^{-1}$; RSD 1.8% and $-2.08 \times 10^{-8}$ $m^2V^{-1}s^{-1}$; RSD 5.3% for the DMPC/SS and POPC/SS liposomes coating, respectively).

[7] (a) Corradini, D.; Cogliandro, E.; D'Alessandro, L.; Nicoletti, I. *J. Chromatogr. A* **2003**, *1013*, 221- 232. (b) Corradini, D.; Bevilacqua, L.; Nicoletti, I. *Chromatographia* **2005**, *62* (in press).

The observed differences in the electro-osmotic flow displayed by DMPC/SS and POPC/SS liposomes coating could be due either to a different charge density on the liposome surface (lower for POPC/SS than DMPC/SS liposomes) owing to a possible different exposition of the cationic counterpart to the external bulk, or to a lower incorporation of the cationic counterpart during the preparation of the liposome composed of POPC and SS, resulting in a lower charge density on the POPC/SS surface.

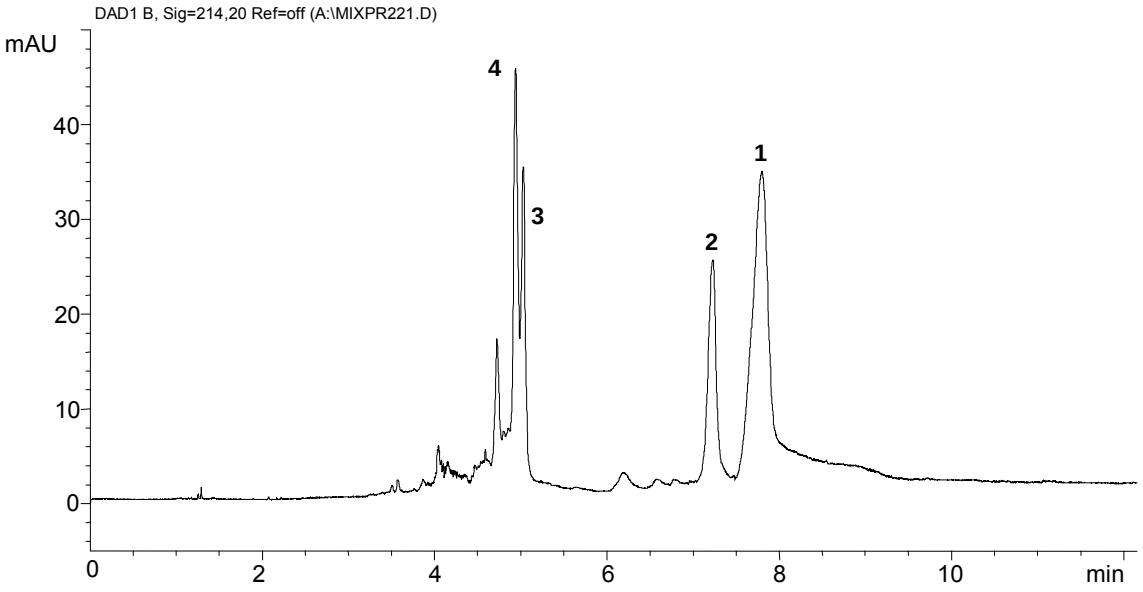



**Figure 5.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated with 60 μM DMPC/ME 9:1 liposomes dispersed in BGE. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 7.4, containing 68 mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

Changing the cationic counterpart SS with its meso form (ME) in the cationic liposome composed of DMPC and cationic gemini surfactant, did not cause appreciable variation of the electrophoretic behaviour of the proteins (Figure 5) and of the electro-osmotic mobility ($-2.82 \times 10^{-8}$ $m^2V^{-1}s^{-1}$; RSD 2.0%) with respect to that observed with the capillary dynamically coated with DMPC/SS 9:1 liposome dispersion (Figure 3). From these findings it can be inferred that in the investigated molecular aggregates, the diastereomeric interactions between the liposome components do not strongly influence the organization of the double layer and the interactions with the macromolecules.

The effect of introducing a plug of cationic liposomes into the capillary tube dynamically coated with POPC/SS 9:1 liposome dispersion is depicted in Figure 6.

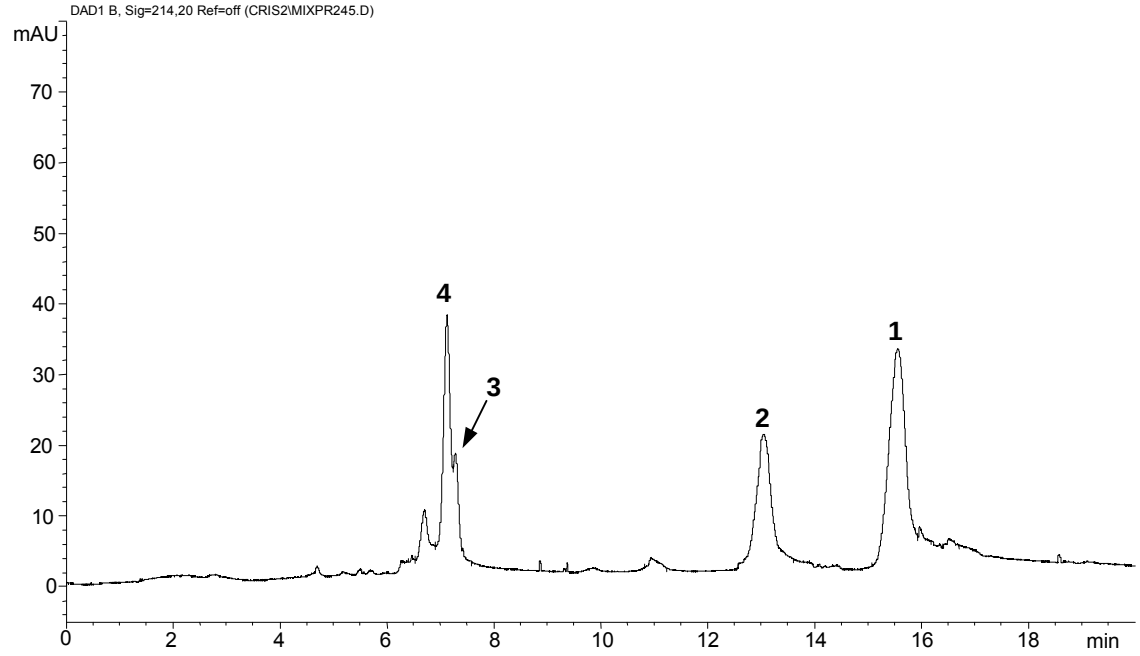



**Figure 6.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated and filled with a plug of 60 μM POPC/SS 9:1 liposomes dispersed in BGE. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

From the electropherogram depicted in Figure 6 it can be noticed that the introduction of a plug of cationic liposomes into the capillary has the only effect to slightly increase the apparent electrophoretic mobility of proteins due to the increased electro-osmotic mobility. The small increase of the electro-osmotic mobility could be due to higher positive charge density immobilized within the Stern region of the electric double layer at the interface between the capillary wall and the electrolyte solution, owing to the increased concentration of cationic liposomes into the capillary tube. Similar results were observed for the introduction of a plug of DMPC/SS 9:1 liposome dispersion into the capillary tube previously dynamically coated with the same liposome dispersion.

These results suggest that either DMPC/SS 9:1 or POPC/SS 9:1 liposomes have a simple masking effect for the silanol groups of the capillary wall rather than selective interactions with basic proteins; the major role in the interactions between basic proteins and cationic liposomes is played by the repulsive electrostatic forces.

### *4.2.3 Effect of the Cationic Gemini Surfactant Counterpart*

The electrophoretic behaviour of the basic proteins in the presence of SS coating on the inner wall of the capillary tube was investigated in order to put in evidence the influence of the cationic gemini surfactant counterpart on the interaction between cationic liposomes and basic proteins.

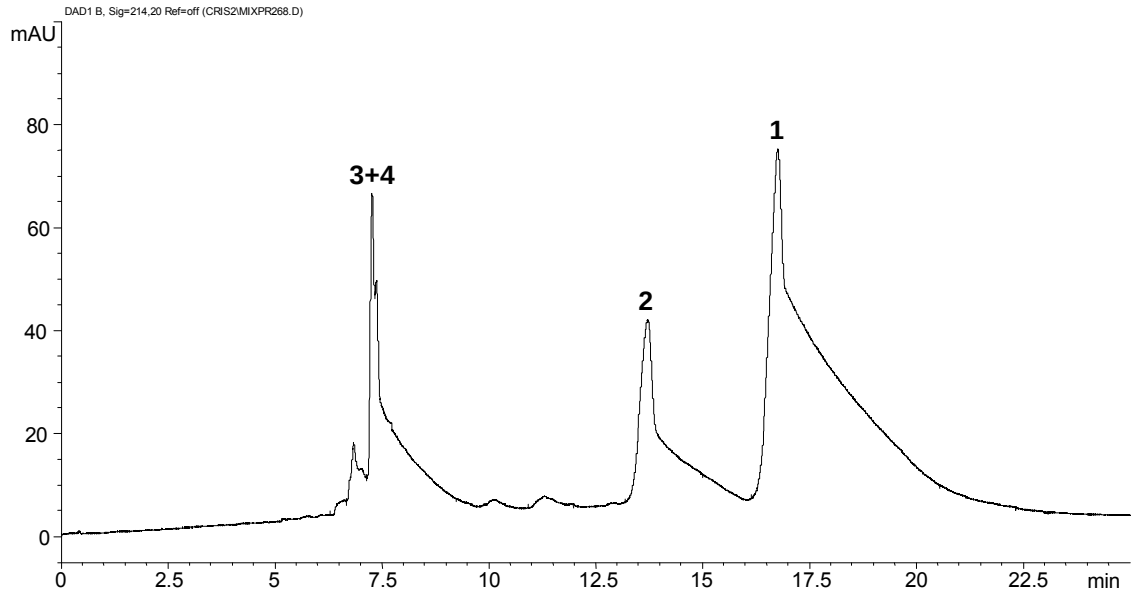



**Figure 7.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary coated with 60 μM SS dissolved in BGE. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

The electropherogram depicted in Figure 7 shows the electrophoretic behaviour of the four basic proteins in the presence of the cationic gemini surfactant SS employed as a coating agent of the capillary wall. At the concentration of 60 μM the cationic gemini surfactant SS is in monomeric form for about 50% being the cmc $2.7 \times 10^{-5}$ M at 321 K.[8] Being the masking effect due to the presence of the cationic liposome adsorbed on the inner wall of the capillary tube, the substitution of the cationic liposomes with the surfactant SS involves variations of the electro-osmotic mobility (reversed electro-osmotic flow; -2.14 $m^2V^{-1}s^{-1}$; RSD 0.7%) similar to those observed with the capillary dynamically coated with DMPC/SS or POPC/SS 9:1 liposome dispersion, but different electrophoretic behaviour of the basic proteins. In fact, in the presence of a SS coating, proteins migrate as broadened peaks, evidencing hydrophobic interactions with the capillary wall coated with SS surfactant.

[8] Unpublished results.

A possible explanation of the hydrophobic interactions involved is depicted in Figure 8.

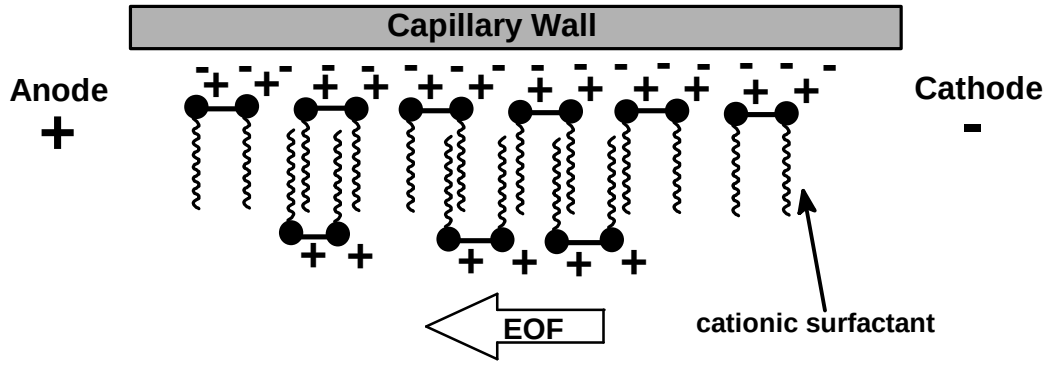



**Figure 8.** Schematic representation of the inner capillary wall in the presence of a cationic surfactant. The cationic surfactant is adsorbed as a monomer on the capillary wall due to the presence of the negatively charged silanol groups.

The absorption of the cationic surfactant SS on the capillary wall can be explained on the basis of the model proposed by Fuerstenau[9] concerning the adsorption of alkylammonium salts on quartz. According to this model, the adsorption in the Stern layer as individual ions of surfactant molecules in dilute solution, results from the electrostatic attraction between the head groups of the surfactant and the ionized silanol groups at the surface of the capillary wall. Van der Wals attraction forces between the hydrocarbon chains of adsorbed and free surfactant molecules in solution may cause their association into hemimicelles, i.e. pairs of surfactant molecules with one cationic group directed toward the capillary wall and the other directed toward solution, giving rise to the structure represented in Figure 8. Proteins could establish either electrostatic or hydrophobic interactions during their migration toward the anode, owing to the particular coating on the inner surface of the capillary wall. Because electrostatic interactions between the positively charged hemimicelles and positively charged proteins are repulsive, the observed peak broadening are expected to be the result of hydrophobic interactions between the hydrophobic tails of either adsorbed or free SS monomers and the hydrophobic moieties of proteins.

[9] Fuerstenau, D.W. *J. Phys. Chem.* **1956**, *60*, 981-985.

### *4.2.4 Influence of pH*

Experiments were carried out in order to investigate how pH could influence the electrophoretic behaviour of the basic proteins in the presence of cationic liposomes composed of POPC/SS 9:1.

From the electropherograms depicted in Figure 9 it can be noticed that the peaks relative to ribonuclease A and α-chymotrypsinogen A are completely resolved in a dynamically coated capillary with a POPC/SS 9:1 liposome dispersion at pH 6.2. The electrophoretic mobility are lower than the mobility observed for the basic proteins at pH 7.4, despite the fact that the value of the electro-osmotic flow is practically unchanged (-2.18 $m^2V^{-1}s^{-1}$; RSD 0.5%) with respect to the value observed in a dynamically coated capillary with POPC/SS 9:1 liposome dispersion at pH 7.4 (-2.08 $m^2V^{-1}s^{-1}$; RSD 0.7%).

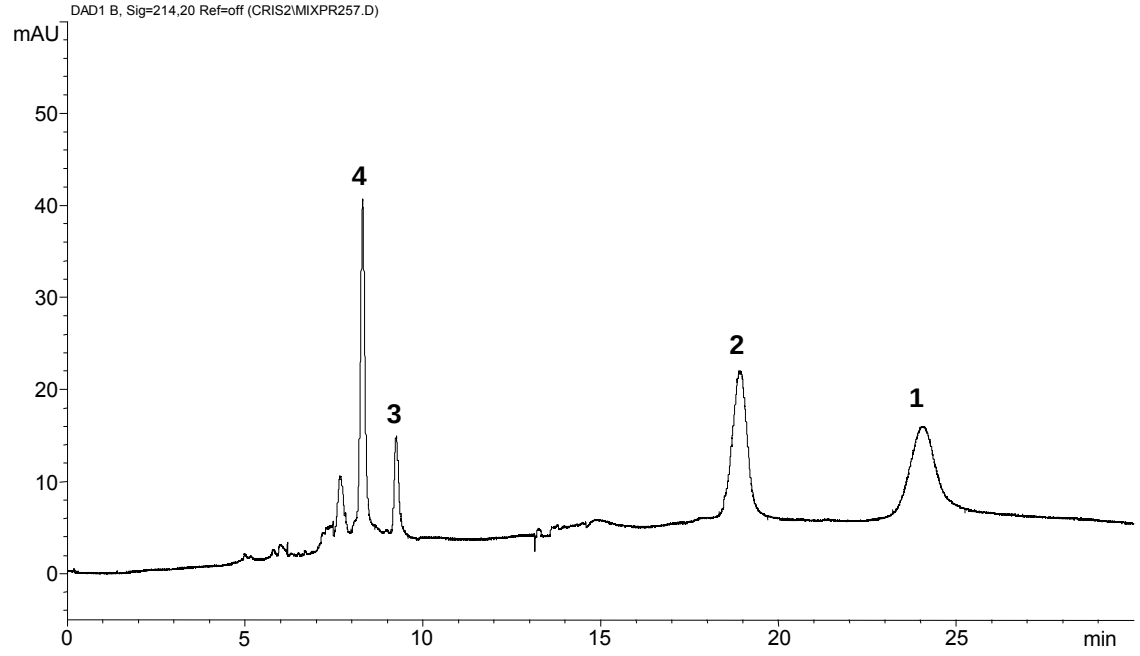



**Figure 9.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated with 60 μM POPC/SS 9:1 liposomes dispersed in BGE at pH 6.2. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 6.2, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

The differences observed in the electrophoretic behaviour of the basic proteins at pH 6.2 with respect to the electrophoretic behaviour of these basic proteins at pH 7.4 are expected to be due to the higher protonation of the aminoacidic residues that makes the proteins more positively charged (changing the charge/size ratio) with a consequent increase of the cathodic electrophoretic mobility.

Filling the capillary with a plug of the POPC/SS 9:1 liposome dispersion, has the effect to increase the electro-osmotic mobility (-2.40 $m^2V^{-1}s^{-1}$; RSD 2.4%) and thus the apparent mobility of the basic proteins is higher (Figure 10).

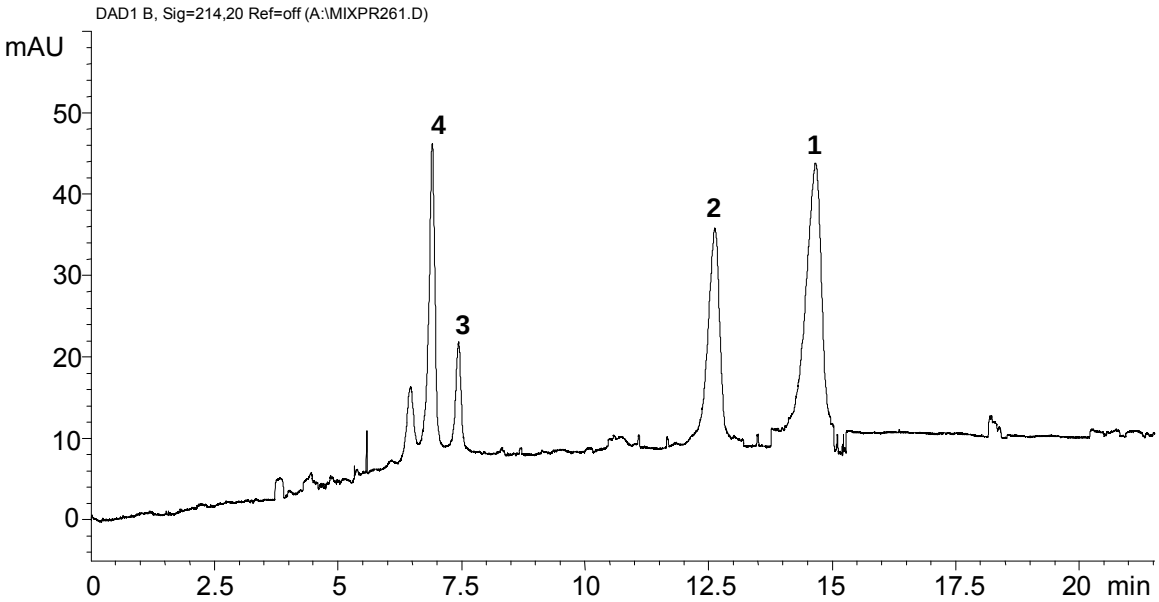



**Figure 10.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated and filled with 60 μM POPC/SS 9:1 liposomes dispersed in BGE at pH 6.2. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 6.2, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

These findings confirm the hypothesis made in the previous experiments performed at pH 7.4 by different operational mode: the interactions between cationic liposome and basic proteins in acidic domains are essentially of repulsive electrostatic forces. The hydrophobic interactions could be involved but are negligible.

### *4.2.5 Effect of Different Molar Ratio of Phospholipid and Cationic Gemini Surfactant*

Experiments on the electrophoretic behaviour of the basic proteins in a dynamically coated capillary with a liposome dispersion of POPC/SS in a molar ratio 6:4 were performed at pH 7.4 in order to investigate the influence of an higher percentage (being the total concentration of lipids constant) of the cationic counterpart in the aggregate.

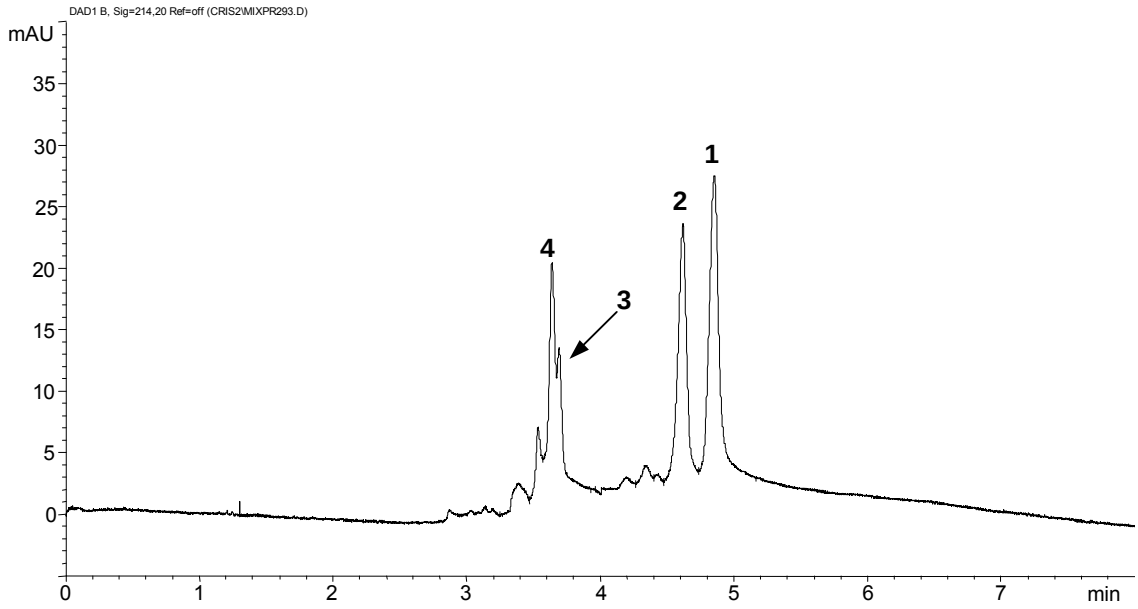



**Figure 11.** Electrophoretic behaviour of the four basic proteins in bare-fused silica capillary dynamically coated with 60 μM POPC/SS 6:4 liposomes dispersed in BGE. Proteins, 1=lysozyme, 2=cytochrome c, 3=ribonuclease A, 4=α-chymotrypsinogen A. Electrolyte solution (BGE), 20mM phosphate buffer, pH 7.4, containing 68mM NaCl. Applied voltage, -10.0 kV; anodic detection at 214 nm.

From the electropherogram depicted in Figure 11 it can be noticed that the apparent electrophoretic mobility of the basic proteins in the dynamically coated capillary with 60 μM POPC/SS 6:4 liposomes dispersed in the BGE are higher than the apparent electrophoretic mobility observed for the basic proteins in the dynamically coated capillary with a 60 μM POPC/SS 9:1 liposomes dispersion. The reason is the higher electro-osmotic mobility (-3.63 $m^2V^{-1}s^{-1}$; RSD 2.8%) due to the increasing positive charge density on the surface of the cationic liposomes adsorbed on the capillary wall, affecting the zeta-potential (section 4.2.1).

### *4.2.6 Circular Dichroism Experiments*

Circular dichroism measurements were carried out in order to confirm the hypothesis made in the previous sections concerning the electrostatic interactions involved between cationic liposomes composed of POPC/SS and basic proteins in different conditions.

A first set of experiments were performed at pH 7.4 with POPC/SS 9:1 liposome dispersion and the four basic proteins under the same concentration conditions (cationic liposome/protein about 1:30) used in the CE experiments.

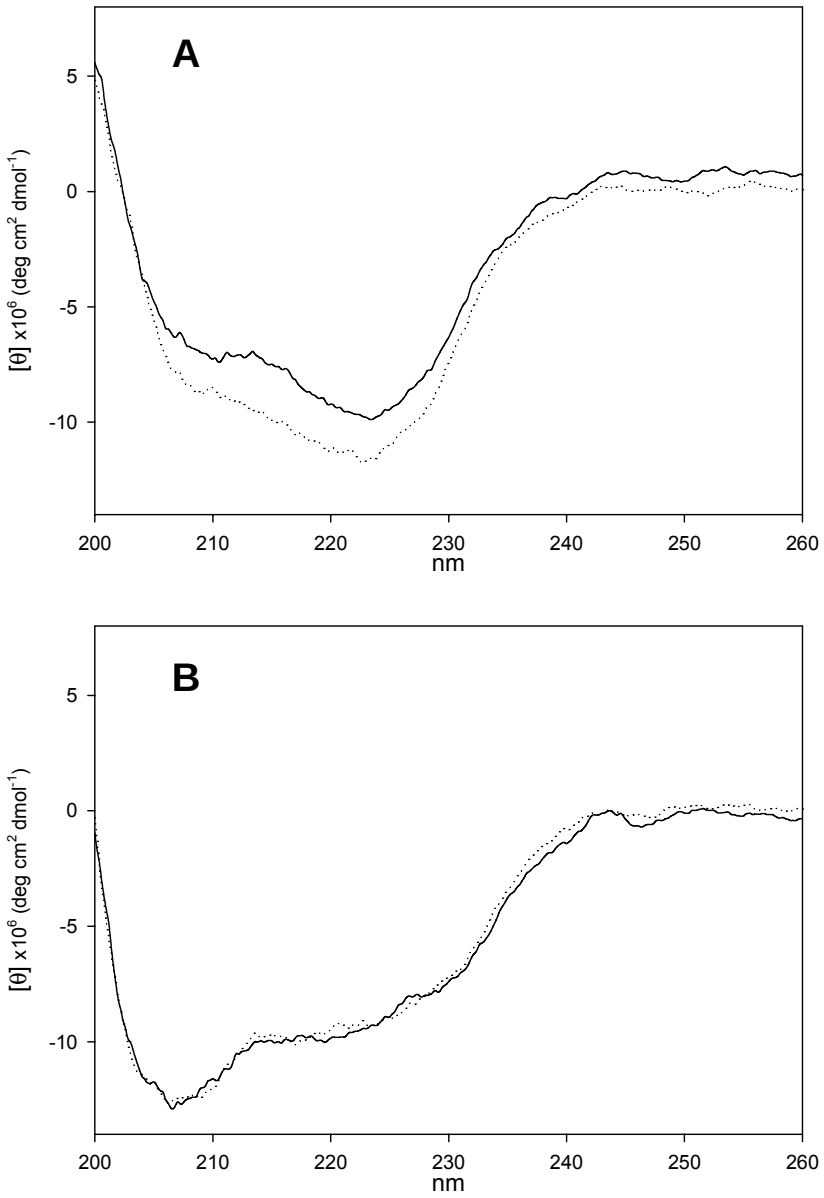



**Figure 12.** Circular dichroism spectra of cytochrome c (A) and lysozyme (B) in the absence (solid line) and in the presence (dotted line) of POPC/SS 9:1 liposome dispersion. Protein/POPC-SS molar ratio, 1:30. Experiments carried out in 20mM phosphate buffer, pH 7.4, containing 68mM NaCl.

From the dichroic bands of cytochrome c and lysozyme plotted in Figure 12, it can be inferred that the interactions between cationic liposomes and basic proteins are essentially electrostatic, owing to unchanged secondary structure of the proteins (as described in section 3.3).

The effect of changing the POPC/SS molar ratio from 9:1 to 6:4 is depicted in Figure 13:

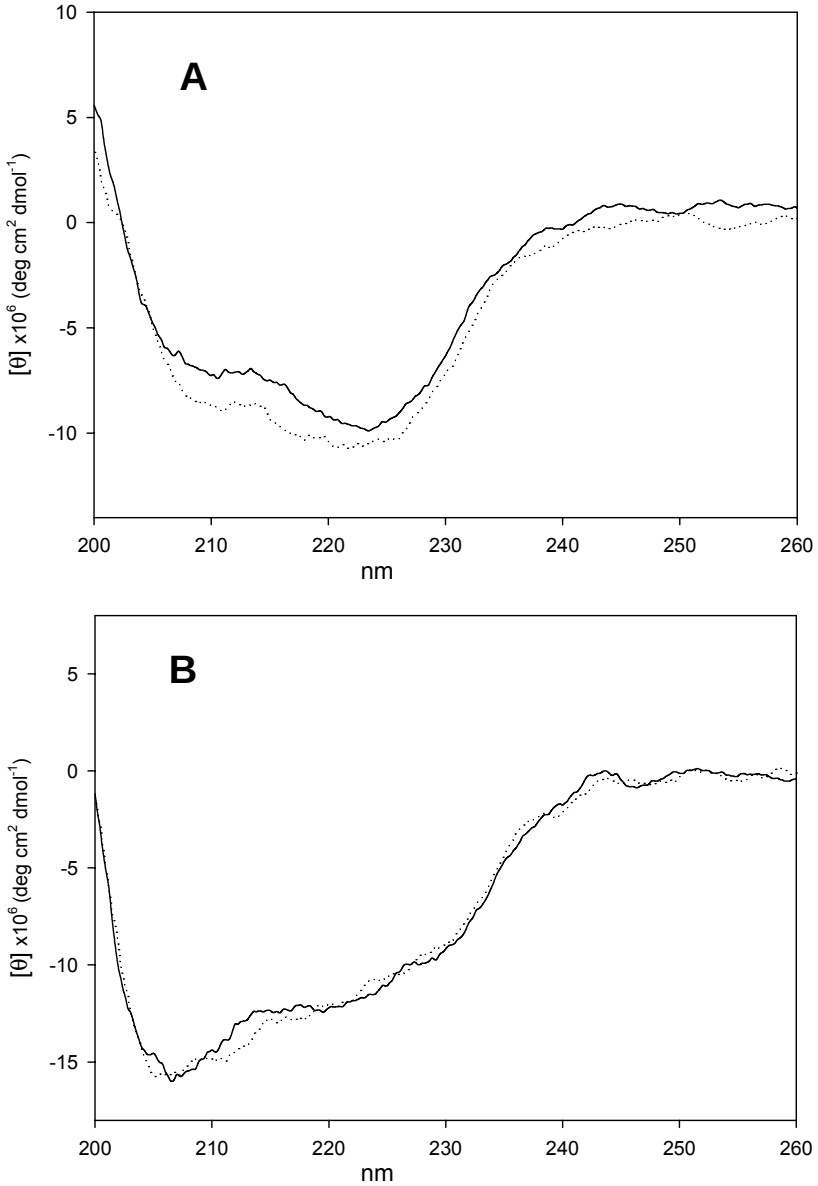



**Figure 13.** Circular dichroism spectra of cytochrome c (A) and lysozyme (B) in the absence (solid line) and in the presence (dotted line) of POPC/SS 6:4 liposome dispersion. Protein/POPC-SS molar ratio, 1:30. Experiments carried out in 20mM phosphate buffer, pH 7.4, containing 68mM NaCl.

The dichroic band relative to the secondary structure of the basic proteins in the presence of the POPC/SS 6:4 liposomes are, as expected, substantially unchanged, as observed from the dichroic band of the proteins in the presence of the cationic liposomes composed of POPC/SS 9:1. This fact further confirms the major role of the electrostatic interactions between both positively charged liposomes and proteins.

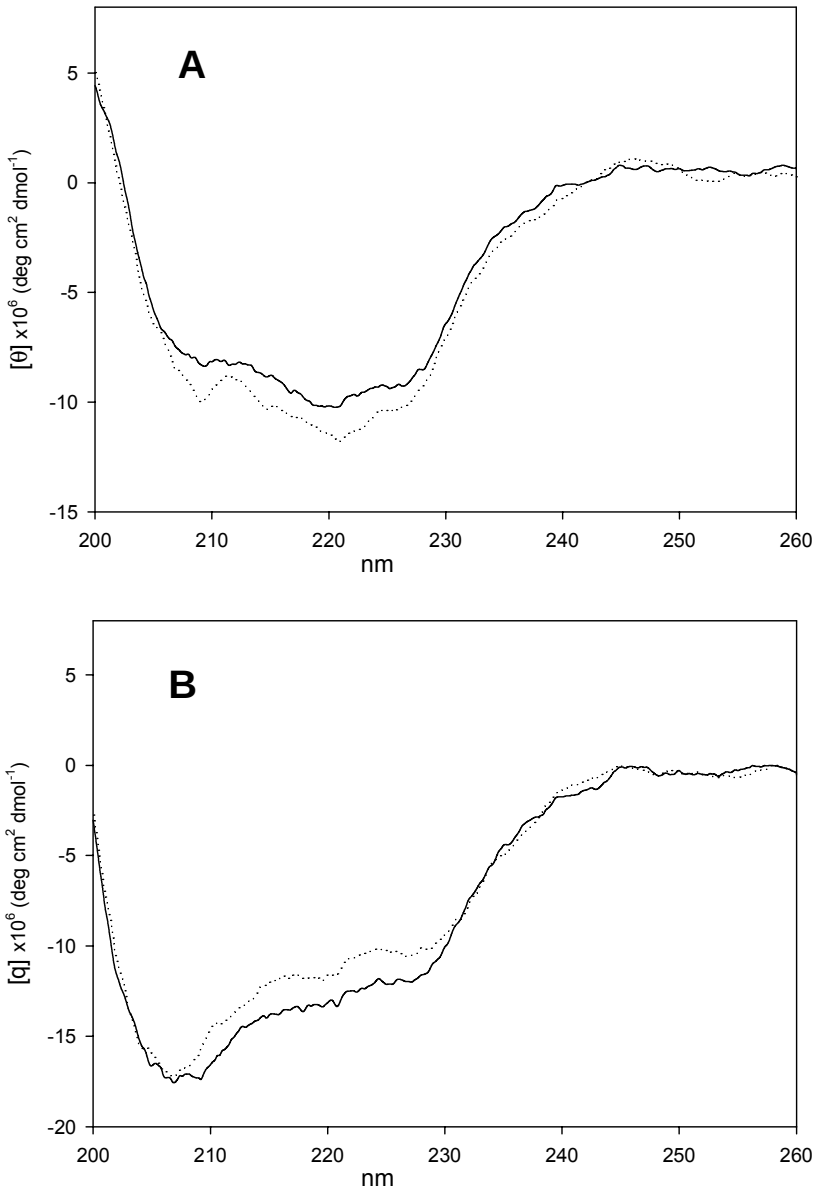



**Figure 14.** Circular dichroism spectra of cytochrome c (A) and lysozyme (B) in the absence (solid line) and in the presence (dotted line) of POPC/SS 9:1 liposome dispersion. Protein/POPC-SS molar ratio, 1:30. Experiments carried out in 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl.

No particular effect can be noticed in the dichroic bands depicted in Figure 14 at pH 6.2. Once again, the unchanged dichroic band relative to the secondary structure of the proteins, put in evidence that electrostatics are the main forces involved in the interactions between cationic liposomes and basic proteins.

Similar behaviour can be observed in the dichroic bands of ribonuclease A and α-chymotrypsinogen A (plot not reported) in the presence of a cationic liposome dispersion.

## *4.3 Conclusions*

In this chapter cationic liposome capillary electrophoresis was performed in order to investigate how the modification of the liposome surface could influence the interactions between cationic liposomes and basic proteins. For the modification of the outer surface of the phospholipid liposomes we used a cationic gemini surfactant mixed in different molar ratio with POPC or DMPC at different pH.

The first observation was that cationic liposomes reversed the electro-osmotic flow from cathodic to anodic indicating that their strong interaction with the inner capillary wall affects the zeta-potential. The resulting coating of the inner capillary wall is demonstrated to be relatively stable and can be completely removed only treating the inner surface of the capillary with a strong alkaline solution.

Concerning the cationic liposome capillary electrophoresis of the basic proteins, it was observed the reversal migration order with respect to the migration order observed in a dynamically coated capillary with zwitterionic liposomes (POPC) because of the reversed electro-osmotic flow. The lower apparent electrophoretic mobility of the basic proteins observed in the presence of a POPC/SS liposomes coating with respect to the mobility observed in the presence of a DMPC/SS liposomes coating at pH 7.4, could be due to a different charge density on the liposome surface (lower for POPC/SS than DMPC/SS) causing a lower electro-osmotic mobility.

Experiments performed using the cationic surfactant counterpart of the cationic liposomes as dynamic coating agent put in evidence either hydrophilic or hydrophobic interactions that the SS surfactant could establish with the basic proteins.

The only differences observed in the electrophoretic behaviour of the basic proteins, lowering the pH from 7.4 to 6.2, were due to the increasing of the protonated amino acid residues changing the charge/size ratio and thus affecting the electrophoretic mobility.

Increasing the percentage of the cationic surfactant on the liposomes has the effect to increase the electro-osmotic mobilities owing to the increase of the positive charge density.

From the electrophoretic behaviour of the basic proteins in the presence of a cationic liposome coating, observed in all conditions (different phospholipids/cationic surfactant molar ratio and pH), it can be inferred that the interactions are mainly electrostatic

because no occurrence in the electrophoretic behaviour of the basic proteins was observed.

Circular dichroism experiments performed in order to confirm the electrostatic interactions involved, point out that the secondary structure of the proteins is not affected by the presence of the cationic liposomes, further corroborating the evidence of the major role played by electrostatics.

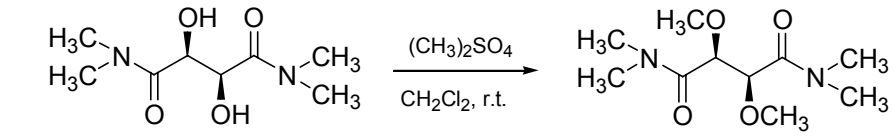
The unchanged secondary structure of the proteins should indicate that they are not denatured by the presence of cationic liposomes. In this respect liposome capillary electrophoresis may play an important role not only as investigative or analytical technique for studying biomolecule-molecular aggregate interactions, but also as preparative method for the recovery of the starting material.

## *4.4 Experimental Section*

### *4.4.1 Preparation of the Cationic Gemini Surfactant SS*

(2*S*,3*S*)-2,3-dimethoxy-1,4-bis(*N*,*N*-dimethylamino)butane, **3,** was prepared by methylation and reduction of the *N*,*N*,*N*′,*N*′-tetramethyl-*L*-tartaramide according to the procedure described by Seebach et al.[10] reported below.

***(2R,3R)-(+)-2,3-dimethoxy-N,N,N′,N′-tetramethylsuccinamide (2)***

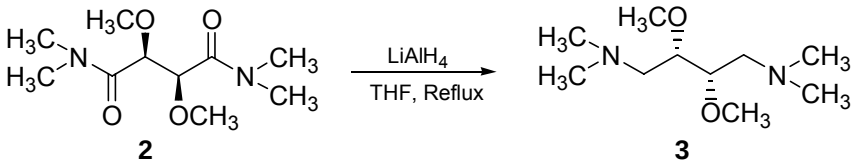



*N,N,N',N'*-tetramethyl-*L*-tartaramide **2**

In a round bottomed flask 10.5 mL of dichloromethane, 2.4 mL of an aqueous solution 50% of sodium hydroxide, 2.3 mL (25 mmoles) of dimethylsulphate and 6 mg of triethylbenzylammonium chloride (TEBA) were introduced. The mixture was kept at room temperature and within 30 minutes (to avoid the decomposition of dimethylsulphate in alkaline solutions) 2g (9.8 mmoles) of *N,N,N′,N′*-tetramethyl-*L*-tartaramide were added in small portions under vigorous stirring. After 40 hrs, 15 mL of an aqueous ammonia solution 1M were added and the mixture was kept stirring for 3.5 hrs. The mixture was extracted with dichloromethane and the combined organic extracts were dried over anhydrous sodium sulphate, filtered and concentrated to give a crude yellow oil that tends to crystallize at 25°C. 1.6g (7 mmoles) of the purified product (yield 70%) were obtained by crystallization from cyclohexane: m.p. 67÷69°C ; $^1$H NMR (δ $CDCl_3$: 4.56 (s, 1H); 3.36 (s, 3H); 3.09 (s, 3H); 2.85 (s, 3H)); $[\alpha]_D^{25}$=+116° (C=3, $EtOH_{abs}$).

[10] Seebach, D.; Kalinowski, H.O.; Bastani, B.; Crass, G.; Daum, H.; Dorr, H.; DuPreez, N.P.; Ehrig, V.; Langner, W.; Nussler, C.; Oei, Hoc-An; Schmidt, M. *Helv. Chim. Acta*, **1977**, *60*, 301-325.

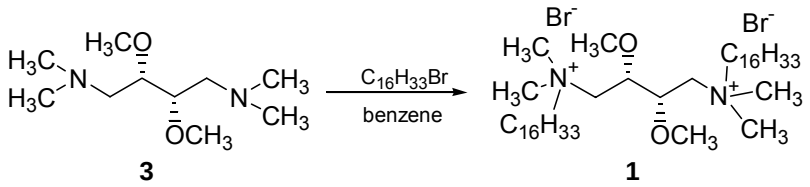
***(2S,3S)-(+)-2,3-dimethoxy-1,4-bis(dimethylamino)butane (3)***




**2** **3**

In a round bottomed flask, 14.5 mL of anhydrous tetrahydrofurane, 612 mg (16 mmoles) of lithium aluminiumhydride and 1.5 g (6.5 mmoles) of (2*R*,3*R*)-(+)-2,3-dimethoxy-*N,N,N',N'*-tetramethylsuccinamide (**2**) were added. After 3.5 hrs of heating at reflux, 0.5 mL of water, 1.5 mL of 10% potassium hydroxide aqueous solution and 0.5 mL of water, were added to the cooled mixture. At the same time, tetrahydrofuran was added under vigorous stirring. The obtained yellow solid was filtered, washed with tetrahydrofurane and the solvent was evaporated to obtain a red crude oil. 1.1 mL (5 mmoles) of the purified colourless oil (yield 75%) were obtained by distillation under reduced pressure, over lithium aluminiumhydride: b.p.=92°C at 9 mmHg; $^1$H NMR (δ $CDCl_3$: 3.43 (s, 4H) ; 2.25-2.47 (m, 8H)); $[\alpha]_D^{25}$=+14.3 (neat); d=0.896g/mL.

***(2S,3S)-2,3-dimethoxy-1,4-bis(N,N-dimethyl-N,-hexadecylammonium)butane dibromide (1)***




**3** **1**

To 0.2 g (1mmol) of **3** in benzene was added 1.2 mL (3.9 mmol)of bromohexadecane and the solution was kept at room temperature. After 20 days the formation of a white precipitate was observed. The reaction mixture was filtered; the precipitate was washed many times with diethyl ether and crystallized from acetone (yield 60%): mp 135°C; $^1$H NMR, (δ $CDCl_3$: 4.691 (s, 1H); 3.934-3.779 (m, 2H); 3.680 (s, 3H); 3.495-3.481 (m, 2H); 3.333 (s, 6H) ; 1.741 (bs, 2H); 1.308-1.210 (m, 26H); 0.836 (t, 3H)); $^{13}$C NMR, (δ $CDCl_3$: 72.71; 65.64; 65.09; 58.35; 52.30-52.20; 31.84; 29.62-29.20; 26.21; 22.84-

22.60; 14.05.); $[\alpha]_D^{25^\circ}$ = -20° (C=3, $EtOH_{abs}$); ES-MS $[735]^+$ (monocation). Anal. ($C_{42}H_{90}N_2O_2Br_2 \cdot 1.5\ H_2O$) C, H, N.

### *4.4.2 Preparation of Cationic Liposomes*

Phospholipids- cationic mixed liposomes consisting of large unilamellar vesicles (LUV) were prepared as previously described (chapter 2, section 2.1). The desired amount of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and (2*S*,3*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane dibromide (**1**), were dissolved in chloroform in a round-bottomed flask. The round-bottomed flask was placed in a rotary evaporator with a water bath at 30-35°C to produce a thin layer of mixed phospholipid surfactant on the inner surface of the flask. The residue traces of the solvent were removed under high vacuum overnight. The resulting thin lipid film was hydrated with the appropriate buffer, under shaking to give a dispersion of multilamellar vesicles (MLV) with a lipid concentration of 10 mM. The MLV dispersion was subjected to five freeze-thaw cycles from liquid nitrogen in order to reduce multilamellarity and processed to LUV by extrusion through a polycarbonate filters of 0.2 and 0.1 μm pore size. First, the dispersion was passed 10 times through a 0.2 μm pore size polycarbonate filter and then 10 times through a 0.1 μm pore size filter. The prepared liposome dispersion was stored at 4°C in the dark.

### *4.4.3 Capillary Electrophoresis*

All experiments were performed using an $HP^{3D}$ Capillary Electrophoresis system from Agilent (Waldbronn, Germany), consisting of a high voltage power supply, a diode array UV–vis detector and an air-cooling device for temperature control of the cartridge containing the capillary tube. The capillary electrophoresis unit was interfaced with an HP Vectra XM 5 166MHz personal computer running the $HP^{3D}$CE ChemStation software, providing system control in addition to data acquisition and evaluation. Capillary tube of 0.050mm I.D. and 0.375mm O.D. was purchased from Quadrex (New Haven, CT, USA). Capillaries having total length of 330mm and distance from the detection window and capillary end of 85mm were prepared in house. Prior to use for the first time, the new fused-silica capillary was flushed successively with 0.5 M

sodium hydroxide (30 min), water (10 min), and 0.5 M hydrochloric acid (30 min), followed by a second treatment with water (10 min), 0.5 M sodium hydroxide (30 min), water (10 min).[11] All experiments were carried out at constant applied voltage of ±10 kV with the temperature of the capillary cartridge set at 25°C. Samples were introduced into the capillary by pressure injection at 5 kPa for 3.0 sec. In the case of anodic electro-osmotic flow the electrode polarity was reversed so that sample introduction and detection took place at the cathodic and anodic end respectively. Before the plug of the liposomes dispersion or analytes injection, the capillary was preconditioned with appropriate buffer which was the background electrolyte solution (BGE) used in the capillary experiments. After preconditioning the capillary with the BGE, the electro-osmotic flow was measured on the basis of the migration time of the neutral marker mesityl oxide.

*Cationic Liposome Capillary Electrophoresis of Basic Proteins*.

In the dynamically coating approach, capillary tube was first rinsed for 5 minutes at 90 kPa with a 60 μM cationic liposomes (CL) in BGE solution and then rinsed with BGE not containing cationic liposomes (i.e. CL-free BGE) in order to remove the liposomes not adsorbed on the capillary wall and to have a dynamic coating on the inner wall of the capillary tube. CL-free BGE was also employed as running electrolyte solution. Between runs capillary was rinsed with the 60 μM CL in BGE for 1.0 min at 90 kPa in order to renewing the coating followed by a 1.0 min at 90 kPa rinse CL-free BGE. In the pseudo stationary phase approach, the capillary was dynamically coated and filled with 60 μM CL in BGE solution. Between runs the capillary was rinsed with 60 μM CL in BGE solution for 1.0 min at 90 kPa, then with BGE for 1.0 min at 90 kPa and finally filled with a plug of 60 μM CL in BGE for 250 sec with pressure of 5 kPa (corresponding to the volume of the capillary tube; section 3.4.2) in order to refresh the coating and to fill again the capillary tube with the CL dispersion.

### *4.4.4 Circular Dichroism*

CD measurements were carried out with a Jasco spectropolarimeter J-715 (Jasco, Easton, MD). Spectra were acquired in a 0.5-cm path length quartz cuvette and were measured as the average of three scans from 200 to 350 nm at a scan rate of 50 nm/min.

[11] Corradini, D.; Cogliandro, E.; D'Alessandro, L.; Nicoletti, I. *J. Chromatogr. A* **2003**, *1013*, 221-232.

CD spectra were registered at $T$ = 298 K immediately after the addition of the liposome dispersion to the solution of the proteins.

### *4.4.5 Chemicals and Samples*

Reagent-grade phosphoric acid, sodium hydroxide, sodium chloride, chloroform were purchased from Carlo Erba (Milan, Italy). Deionized water was obtained by a Milli-Q water purification system from Millipore (Bedford, MA, USA) and degassed by sonication before use. Mesityl Oxide was obtained by Fluka (Milan, Italy). Cytochrome c (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas) and α-chymotrypsinogen A (from bovine pancreas), from Sigma (Milan, Italy), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dimiristoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*,*N*,*N'*,*N'*-tetramethyl-*L*-tartaramide was purchased from Aldrich (Milan, Italy) and bromohexadecane from Fluka (Milan, Italy).

### *4.4.6 Buffers*

Buffers consisting of phosphoric acid and its sodium salt were prepared by titrating the proper amount of requested acid to the desired pH value with 1.0 M sodium hydroxide. The pH was measured with a glass electrode Model 52–02 and Model Basic 20 pH-Meter, both from Crison (Alella, Spain). All solutions were filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sonication before use.

# Chapter 5- *Chiral Recognition of Dipeptides in Biomembrane Models*

## *5.1 Introduction*

The study of chiral interactions between membrane models and chiral solutes, can be useful in many research fields. The organization of biomembranes is based on molecular recognition phenomena, and the comprehension of the role of chiral recogniton could be useful in the elucidation of their structure and organization, being biomembranes formed by enantiopure lipids and proteins. Moreover chiral recognition phenomena play a fundamental role also in the interactions between biomembranes and other biomolecules. The comprehension of the interactions involved in the organization of these systems, may be useful either for the advancement of predictive recognition models for many compounds or, for the development of new chiral stationary phases for enantioseparations,[1] for the development of new sensors[2] for the detection of the optical purity of a compound, or for designing effective asymmetric catalysts. The use of CE has for sure the double valence of investigating the interactions responsible of chiral recognition in biomembrane models and of exploring new chiral phases for analytical separation of enantiomers.[3]

In this chapter, after a brief illustration of the separations principles of micellar electrokinetic chromatography (MEKC), an investigation on chiral recognition of dipeptides in chiral micellar aggregates, will be reported.

[1] Maier, N.M.; Schefzick, S.; Lombardo, G.M.; Feliz, M.; Rissanen, K.; Lindner, W.; Lipkovitz, K.B. *J. Am. Chem. Soc*. **2002**, *124*, 8611-8629.

[2] (a) Kubo, Y.; Hirota, N.; Maeda, S.; Tokita, S. *Analytical Science*, **1998**, *14*, 183-189. (b) Xu, M.; Lin, J.; Hu, Q.; Pu, L. *J. Am. Chem. Soc*. **2002**, *124*, 14239-14246. (c) Tsukabi, K.; Nuruzzaman, M.; Kusumoto, T.; Hayashi, N.; Bin-Gui, W.; Fuji, K. *Org. Lett*. **2001**, *3*, 4071-4073.

[3] (a) Chankvetadze, B. *Electrophoresis* **2002**, *23*, 4022-4035. (b) Bo, T.; Wiedmer, S.K.; Riekkola, M.L. *Electrophoresis* **2004**, *25*, 1784-1791.

## *5.2 Micellar Electrokinetic Chromatography (MEKC)*[4]

### *5.2.1 Principle of Separation in Micellar Electrokinetic Chromatography (MEKC)*

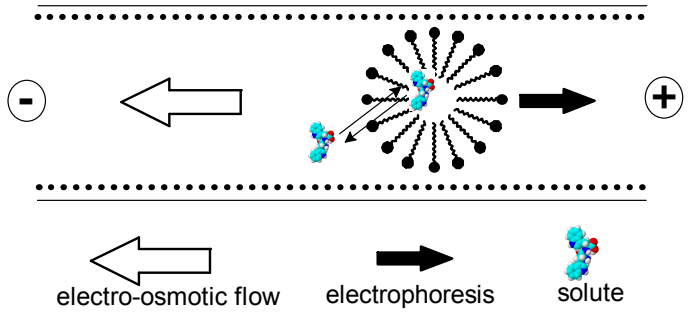



**Figure 1.** Schematic illustration of the separation principles of the MEKC.

The separation principle of MEKC is schematically shown in Figure 1. A bare fused silica capillary is filled with an anionic surfactant solution, in which the concentration of the surfactant is higher than its critical micelle concentration (cmc), so that micelles are formed. When an anionic surfactant is employed, the micelle is forced to migrate toward the positive electrode (anode) by electrophoresis. The electro-osmotic flow migrates toward the negative electrode (cathode) owing to the negative charge of the capillary surface. The electro-osmotic flow is larger than the electrophoretic migration of the micelle under neutral or basic conditions, and therefore, the anionic micelle also migrates toward the cathode at a retarded velocity.

When a neutral analyte is injected into the micellar solution, it will be distributed between the micelle and bulk solution. The analyte will migrate with the same mobility of the micelle ($\mu_{mc}$) when it is incorporated into the micelle, and with the electro-osmotic mobility ($\mu_{eo}$), when it is in the bulk solution. Thus the electrophoretic mobility ($\mu_R$) of the analyte depends on the distribution coefficient of the analyte into the micelle. In other words, the migration time of the analytes, $t_R$, is in the range between the migration time of the bulk solution, $t_{eo}$, and of the micelle, $t_{mc}$ (Figure 2).

[4] Otsuka, K.; Terabe, S. *Micellar Electrokinetic Chromatography* in Methods in Molecular Biology, vol. 52: Capillary Electrophoresis. Edited by K. Altria, Humana Press Inc., Totowa, NJ.

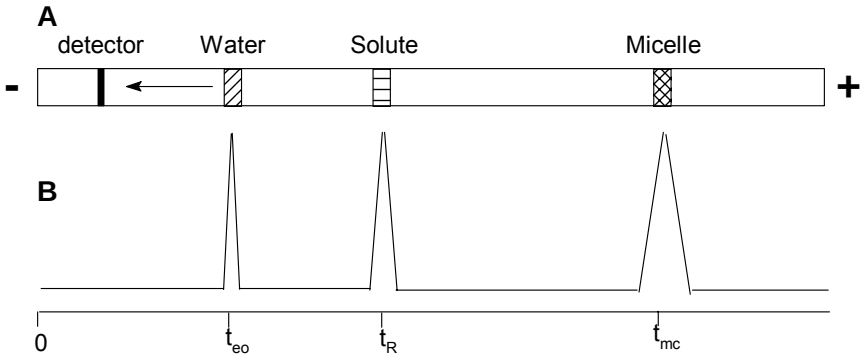



**Figure 2.** Schematic representation of the zone separation in MEKC (A) and the corresponding electropherogram (B).

### *5.2.2 Capacity Factors*

Capacity factor, $k'$, can be defined in the case of conventional chromatography:

$$k' = \frac{n_{mc}}{n_{aq}} \qquad \text{(eqn. 1)}$$

where $n_{mc}$ and $n_{aq}$ are the amount of analyte incorporated into the micelle and in the aqueous solution respectively. Then we can obtain the relationship between the capacity factor and the migration time as:

$$k' = \frac{t_R - t_{eo}}{t_{eo}(1 - \frac{t_R}{t_{mc}})} \qquad \text{(eqn. 2)}$$

It can be written as:

$$t_R = \left[ \frac{1 + k'}{1 + \left( \frac{t_{eo}}{t_{mc}} \right) \cdot k'} \right] \cdot t_{eo} \qquad \text{(eqn. 3)}$$

The value of $t_{mc}/t_{eo}$ can be regarded as a parameter representing the migration time window.

If a neutral analyte is not incorporated into the micelle or does not interact with the micelle at all, the migration time of such a solute is equal to $t_{eo}$ and hence $k'=0$. On the other hand, when the analyte is totally incorporated into the micelle, the migration time window becomes $t_R$, and $k'=\infty$. Thus, the migration time window is limited between $t_{eo}$ and $t_{mc}$.

According to equation 2 $t_{eo}$, $t_R$ and $t_{mc}$ are required to obtain the capacity factor. As a marker of the electro-osmotic flow or $t_{eo}$, methanol is transparent to UV, it can be detected as a baseline deflection with a UV detector owing to a refractive index change. An highly hydrophobic compound which is completely incorporated into the micelle is employed as a marker for micelle ($t_{mc}$).

### *5.2.3 Distribution Coefficient*

The capacity factor can be related to the distribution coefficient, K, between the micelle and the aqueous phase as:

$$k' = K \cdot \frac{V_{mc}}{V_{aq}} \qquad \text{(eqn. 4)}$$

where, $V_{mc}$ and $V_{aq}$ are the volume of the micelle and the aqueous phase, respectively. The phase ratio, $V_{mc}/V_{aq}$, can be written by using the concentration of the surfactant, $C_{sf}$, the critical micelle concentration, cmc and specific volume of the micelle, ν as:

$$\frac{V_{mc}}{V_{aq}} = \frac{\upsilon(c_{sf} - cmc)}{1 - \upsilon(c_{sf} - cmc)} \qquad \text{(eqn. 5)}$$

At low micellar concentration, equation 6 can be written as:

$$k' = K\upsilon(c_{sf} - cmc) \qquad \text{(eqn. 6)}$$

This reveal that the capacity factor increase with an increase in the surfactant concentration.

### *5.2.4 Thermodynamic Parameters*

The distribution coefficient measured at different temperature should follow the van't Hoff equation:

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \qquad \text{(eqn. 7)}$$

where $\Delta H^0$ is the enthalpy change associated with micellar solubilization or the transfer of the solute from the aqueous phase to the micelle, $\Delta S^0$ the corresponding entropy change, R the gas constant and T the absolute temperature. Thus equation 7 allow the calculation of the enthalpy and entropy changes in micellar solubilization from the temperature dependence of the distribution coefficient K.

Combining equation 4 and 7 a relationship between capacity factor k' and the thermodynamic parameters can be derived:

$$\ln k' = -\frac{\Delta H^0}{RT} + \left( \frac{\Delta S}{R} + \ln \frac{V_{mc}}{V_{aq}} \right) \qquad \text{(eqn. 8)}$$

Thus equation 8 allow the calculation of the enthalpy and entropy changes in micellar solubilization from the temperature dependence of the capacity factor k'.

## *5.3 Results and Discussion*

### *5.3.1 Enantiodiscrimination of Dipeptides in Chiral Micellar Aggregates*

We explored an electrophoretic approach to the investigation of chiral recognition in biomembrane models and choose a model previously investigated by $^1$H NMR,[5] namely micelles formed by sodium *N*-dodecanoyl-L-prolinate (**1**) as biomembrane model and the four diastereomers of ditryptophan (**2**) as probes of chirality. Aromatic peptides are suitable for an ease UV detection in CE.

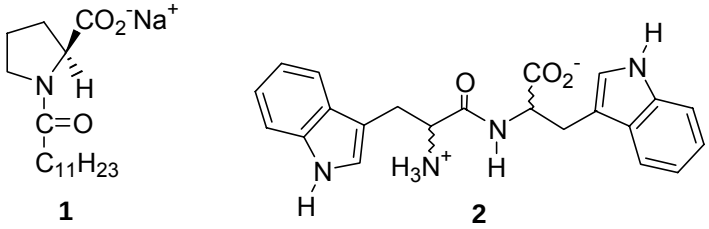


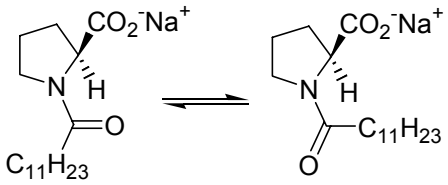

**Figure 3.** Sodium *N*-dodecanoyl-L-prolinate (SDP) (**1**) and ditryptophan (**2**).

Sodium *N*-dodecanoyl-L-prolinate (SDP), though is not a natural amphiphile, is characterized by a high extent of organization and the interactions involved in its micellar aggregates are similar to those responsible for the aggregation and organization of the aggregates formed by natural lipids. In fact it is known that, under aggregating conditions, the geometrical isomers (scheme 1) of amidic surfactants form domains on

[5] Bombelli, C.; Borocci, S.; Lupi, F.; Mancini, G.; Mannina, L.; Segre, A.L.; Viel, S. *J. Am. Chem. Soc.* **2004**, *126*, 13354-13362.

the basis of their E/Z configuration.[6] Moreover, the aggregates formed by SDP are able to discriminate enantiomers.[7]

***Scheme 1***




**3Z** **3E**

### *5.3.2 MEKC of the Homochiral Enantiomers of Ditryptophan in the Presence of Sodium N-dodecanoyl-L-prolinate.*

Experiments were performed in order to investigate the electrophoretic behaviour of the two homochiral enantiomers of ditryptophan, LL-Trp and DD-Trp in the presence of chiral micellar aggregates formed by SDP. Experiments were performed at pH 6.2 in phosphate buffer. This pH was selected on the basis of the lower pH of the phosphate buffering capacity, close to the isoelectric point of the peptides, in order to improve their association with the micelles. LL-Trp and DD-Trp were detected at 280 nm ($\lambda_{max}$) due to strong absorption of the amidic bond of SDP in the UV spectrum region 200-240 nm.

As a preliminary experiment the two homochiral enantiomers were injected into the capillary tube in the absence of a SDP in the electrolyte solution filled into the capillary (Figure 4).

[6] (a) Borocci, S.; Mancini, G.; Cerichelli, G.; Luchetti, L. *Langmuir* **1999**, *15*, 2627-2630. (b) Cerichelli, C.; Luchetti, L.; Mancini, G. *Langmuir*, **1997**, *13*, 4767-4769.

[7] (a) Belogi, G.; Croce, M.; Mancini, G. *Langmuir*, **1997**, *13*, 2903-2904. (b) Bella, J.; Borocci, S.; Mancini, G. *Langmuir* **1999**, *15*, 8025-8031.

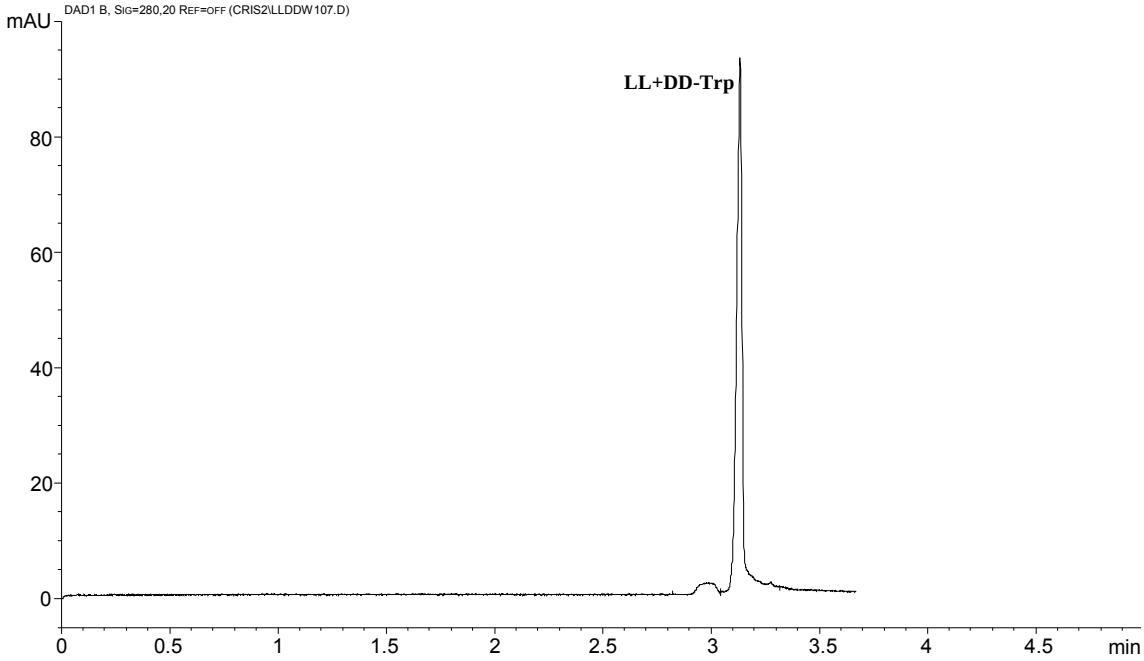



**Figure 4.** Electrophoretic behaviour of LL-Trp and DD-Trp in the absence of chiral micellar aggregates formed by SDP filled into the capillary tube. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 280 nm.

The electropherogram depicted in Figure 4 shows the electrophoretic behaviour of LL-Trp and DD-Trp in the absence of chiral micellar aggregates formed by SDP dispersed in the BGE filled in the capillary tube. As expected, in the absence of a chiral media in the capillary tube LL-Trp and DD-Trp have the same electrophoretic behaviour.

A solution containing the two homochiral enantiomers of ditryptophan, was injected in the capillary tube previously filled with a solution of 100 mM SDP[8] dispersed in 20 mM phosphate buffer at pH 6.2, containing 68 mM NaCl, also employed as background electrolyte. The electro-osmotic ($\mu_{eo}$) mobility and the electrophoretic mobility of the micelles ($\mu_{mc}$) were detected by adding 10% of a methanolic[9] solution of phenanthrene to the LL-Trp and DD-Trp mixture. Phenanthrene is an highly hydrophobic compound and it is assumed to be totally incorporated into the micelle.

[8] To ensure the predominance of micellar aggregates being the cmc of SDP 10 mM: Borocci, S.; Mancini, G.; Cerichelli, G.; Luchetti, L. *Langmuir* **1999**, *15*, 2627-2630.

[9] Methanol is usually not detected by the absorption of UV light but absorbs here due to the slight change of the refractive index.

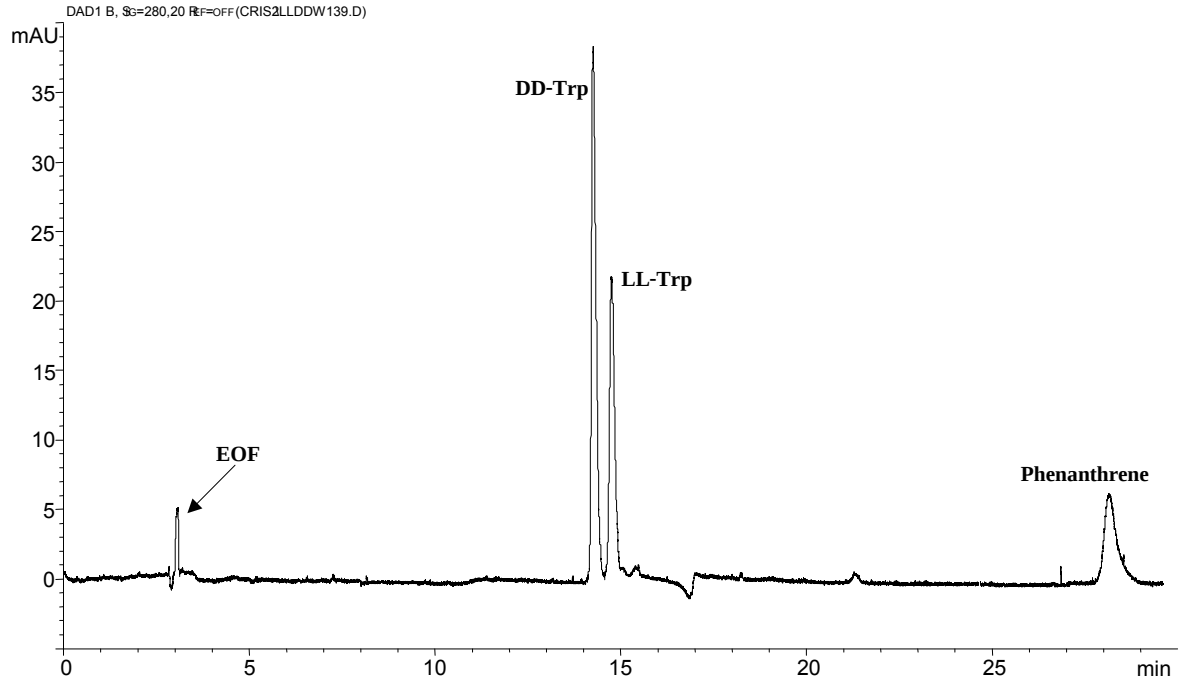



**Figure 5.** Electrophoretic behaviour of LL-Trp and DD-Trp in the presence of chiral micellar aggregates formed by 100 mM SDP dispersed in the BGE filled into the capillary tube. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 280 nm.

From the electropherogram reported in Figure 5, a complete separation of LL-Trp and DD-Trp was observed, suggesting a selective interactions between the chiral micellar aggregates formed by SDP and the two homochiral enantiomers of ditryptophan.

The capacity factors of the homochiral enantiomers of ditryptophan in the presence of chiral micellar aggregates formed by SDP were calculated according to equation 2 in section 5.2.2. These factors refer to the equilibrium

$$\text{peptide} + \text{D} \leftrightarrows \text{peptide}\cdot\text{D}$$

where D is the micellized surfactant. The capacity factors ($k'$) relative to the LL and DD enantiomers of ditryptophan are reported in Table 1.

**Table 1.** Capacity factors of LL-Trp and DD-Trp in the presence of micellar aggregates formed by SDP at increasing concentrations.

| [SDP], mM | $k'_{LL}$ | $k'_{DD}$ |
|---|---|---|
| 0 | 0 | 0 |
| 10 | 0.9 | 0.8 |
| 20 | 2.0 | 1.8 |
| 40 | 4.3 | 3.8 |
| 80 | 8.5 | 7.6 |
| 100 | 10.1 | 9.2 |

As shown in Table 1, the capacity factors increase by increasing the concentration of the surfactant into the capillary tube and the background electrolyte solution.

The different affinity of the homochiral enantiomers of ditryptophan for micellar aggregates of SDP, being the affinity of LL enantiomer higher than that of DD, is in agreement with literature data.[5]

Plotting the values of $k'$ versus the concentration of SDP gives a linear relationship in the concentration range from 10 to 100 mM (Figure 6).

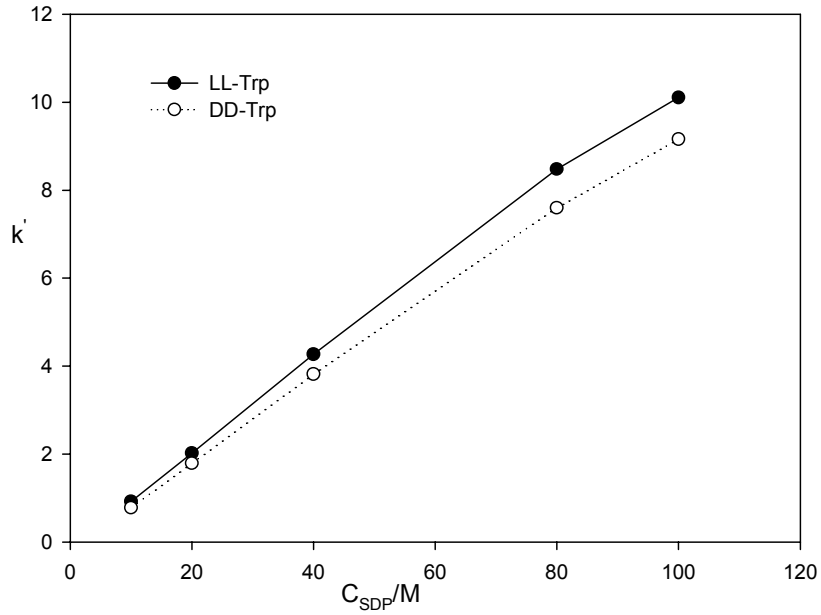



**Figure 6.** Dependence of the capacity factor $k'$ of LL (black circles and solid line) and DD (white circles and dotted line) of ditryptophan from the concentrations of SDP.

This finding implies that the capacity factors remain constant at least at SDP concentration below 100 mM. It was not possible to explore higher concentration of SDP surfactant due to an excessive current intensity because of high ionic concentration.

### *5.3.3 MEKC of the Homo and Heterochiral Enantiomers of Ditryptophan in the Presence of Sodium N-dodecanoyl-L-prolinate.*

Experiments were performed in order to investigate the electrophoretic behaviour of the four diasteromers of ditryptophan, LL, DD, LD and DL-Trp in the presence of chiral micellar aggregates formed by SDP.

First the two enantiomeric couples were injected into the capillary tube in the absence of chiral micellar aggregates formed by SDP dispersed in the BGE filled into the capillary tube.

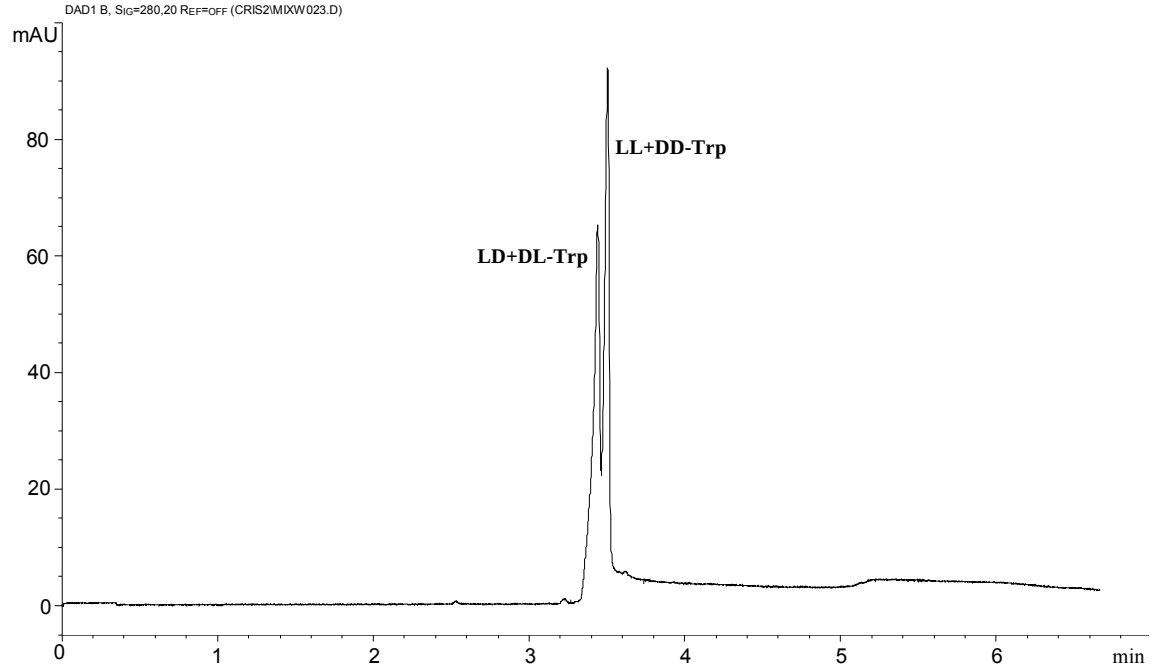



**Figure 7.** Electrophoretic behaviour of the four diastereomers of ditryptophan, LL-Trp, DD-Trp, LD-Trp and DL-Trp in the absence of chiral micellar aggregates formed by SDP. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 280 nm.

From the electropherogram reported in Figure 7 the partial achiral separation of the two pairs of diasteroisomers, exhibiting slightly different electrophoretic behaviour, is

believed arising from the different molecular conformation and charge distribution of the two diasteroisomeric pairs.

A solution containing the four diastereomers of ditryptophan, methanol and phenanthrene as electro-osmotic flow and micelle marker respectively, was then injected in the capillary tube previously filled with a solution of 100 mM SDP dispersed also employed as background electrolyte.

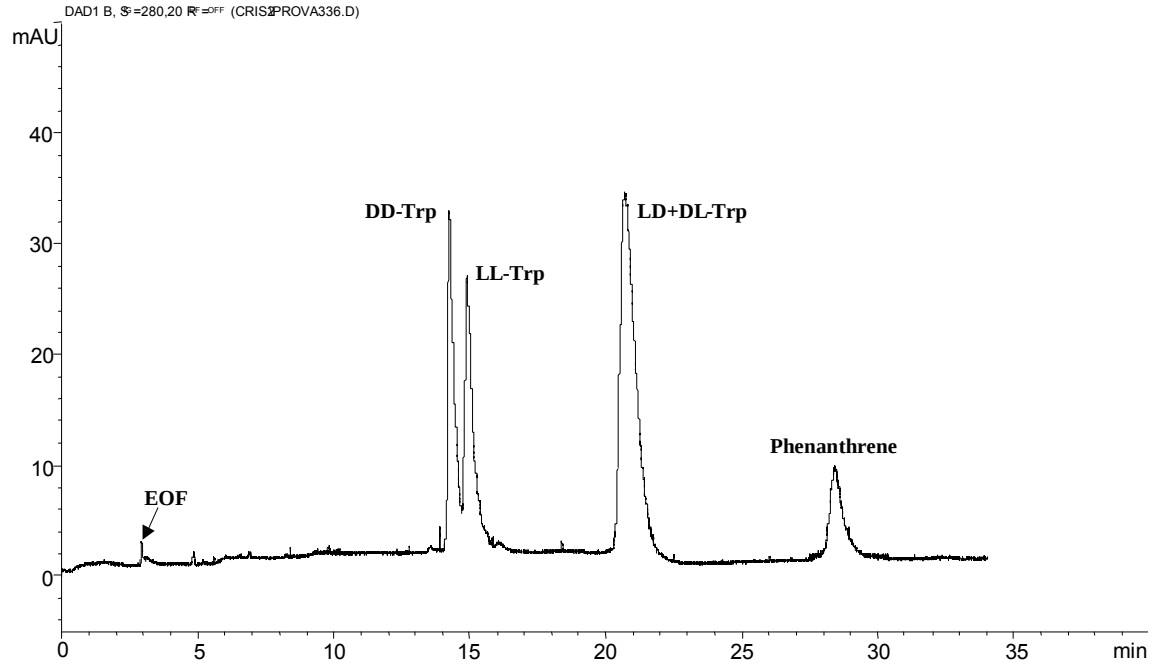
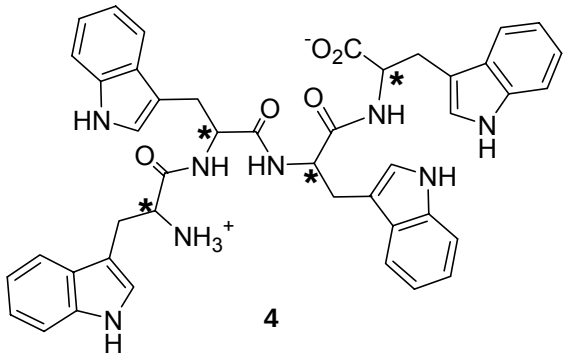



**Figure 8.** Electrophoretic behaviour of the four diastereomers of ditryptophan, LL-Trp, DD-Trp, LD-Trp and DL-Trp in the presence of chiral micellar aggregates formed by 100 mM SDP dispersed in the BGE. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 280 nm.

From the electropherogram depicted in Figure 8 it can be observed that LL and DD-Trp are completely separated as previously shown in Figure 5, whereas LD and DL-Trp display similar electrophoretic behaviour. The calculated capacity factor for the heterochiral enantiomers (k'=28.4 at concentration of SDP 100 mM) shows that heterochiral dipeptides have higher affinity for the chiral micellar phase with respect to the homochiral isomers ($k'_{LL}$=10.1 and $k'_{DD}$=9.2); the association of LD and DL enantiomers with SDP aggregates being stronger than that of DD and LL enantiomers. The higher affinity of LL enantiomer with respect to DD and the higher affinity of the heterochiral enantiomers with respect to the homochiral ones, are in agreement with previously reported results.[5]

### *5.3.4 MEKC of the Homochiral Enantiomers of Tetratryptophan in the Presence of Sodium N-dodecanoyl-L-prolinate.*

The electrophoretic behaviour of the two homochiral enantiomers of tetratryptophan (**4**) was then evaluated.




**4**

**Figure 9.** Tetratryptophan (**4**); (*) indicates the asymmetric carbons.

A solution containing LLLL, DDDD-Trp, methanol and phenanthrene was injected in the capillary tube previously filled with 100 mM SDP dispersed in 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl, also employed as background electrolyte.

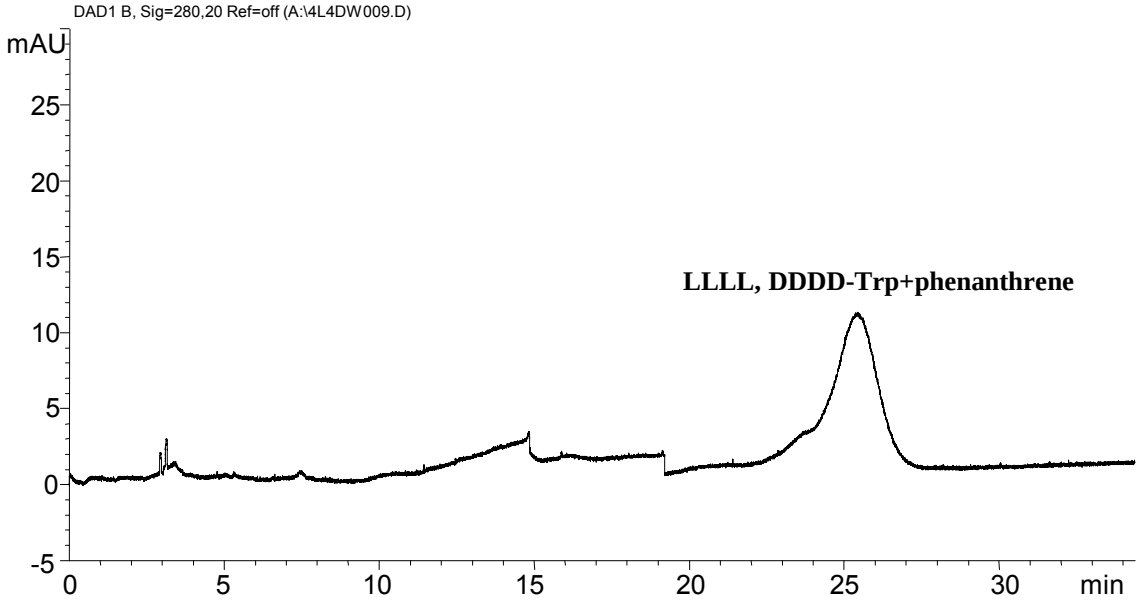



**Figure 10.** Electrophoretic behaviour of the two enantiomers of tetratryptophan in the presence of chiral micellar aggregates formed by 100 mM SDP dispersed in the BGE. Electrolyte solution (BGE), 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; applied voltage, 10.0 kV; cathodic detection at 280 nm.

From the electropherogram reported in Figure 10 it can be noticed that the two enantiomers of tetratryptophan migrate with the same migration times of the mobility

marker of the micelle ($k'=\infty$ because $t_R=t_{mc}$ section 5.2.2) and that no chiral discrimination is observed. From this finding it can be inferred that the two enantiomers of tetratryptophan deeply penetrate in the hydrophobic region of the aggregate as a consequence of their hydrophobicity.

### *5.4 Conclusions*

The interactions between chiral micellar aggregates formed by SDP and the four diastereomers of ditryptophan were investigated by CE and the results were compared with those investigated by $^1$H NMR reported in the literature.[5] The differences observed by NMR in the association of diastereomers of ditryptophan are strongly supported by the electrophoretic investigation.

The homochiral enantiomers of ditryptophan showed a different electrophoretic behaviour, whereas LD and DL heterochiral enantiomers showed the same electrophoretic behaviour. The observed migration behaviour of heterochiral enantiomers in MEKC indicate their higher higher affinity for SDP micelles with respect to the homochiral enantiomer couple, from which it can be inferred their higher hydrophobicity. These findings support the results previously obtained by NMR,[5] that yielded different binding constants for LL and DD enantiomers and the same, higher, binding constant for heterochiral enantiomers. Moreover NMR results suggested a hydrophobic site of binding for heterochiral enantiomers and a location in the interfacial region for homochiral ones.[5]

The electrophoretic investigation performed on the homochiral enantiomeric of tetratryptophan evidenced that the two enantiomers have the same electrophoretic mobility of the micelles. This finding suggests that these high hydrophobic solutes deeply penetrate into the hydrophobic core of the micelle being their capacity factors infinite ($t_R=t_{mc}$ section 5.2.2).

The results obtained by our electrophoretic approach to the study of chiral recognition of dipeptides in biomembrane models are in full agreement with those previously observed by $^1$H NMR experiments,[5] and demonstrated once more that capillary

electrophoresis can be an additional useful technique in the comprehension of chiral recognition phenomena.[10]

[10] (a) Maier, N.M.; Franco, P.; Lindner, W. *J. Chromatogr. A* **2001**, *906*, 3-33. (b) Chankvetadze, B.; Lindner, W.; Scriba, G.K.E. *Anal. Chem*. **2004**, *76*, 4256-4260. (c) Lammerhofer, M.M.; Zarbl, E.; Lindner, W. *J. Chromatogr. A* **2000**, *892*, 509-521. (d) Schug, K.A.; Lindner, W. *Chem. Rev*. **2005**, *105*, 67-113.

## *5.5 Experimental Section*

### *5.5.1 Capillary Electrophoresis*

All the experiments were performed using an HP$^{3D}$ Capillary Electrophoresis system from Agilent (Waldbronn, Germany), consisting of a high voltage power supply, a diode array UV–vis detector and an air-cooling device for temperature control of the cartridge containing the capillary tube. The capillary electrophoresis unit was interfaced with an HP Vectra XM 5 166MHz personal computer running the HP$^{3D}$CE ChemStation software, providing system control in addition to data acquisition and evaluation. Capillary tube of 0.050mm I.D. and 0.375mm O.D. was purchased from Quadrex (New Haven, CT, USA). Capillaries having total length of 330mm and distance from the detection window and capillary end of 85mm were prepared in house. Prior to use for the first time, the new fused-silica capillary was flushed successively with 0.5 M sodium hydroxide (30 min), water (10 min), and 0.5 M hydrochloric acid (30 min), followed by a second treatment with water (10 min), 0.5 M sodium hydroxide (30 min), water (10 min).[11] All the experiments were carried out at constant applied voltage of 10 kV with the temperature of the capillary cartridge set at 25°C. Samples were introduced into the capillary by pressure injection at 5 kPa for 3.0 sec. Before the experiments performed with chiral micellar aggregates, capillary was preconditioned with BGE and the electro-osmotic flow was measured on the basis of the migration time of the neutral marker mesityl oxide.

Concerning the experiments with the chiral micellar aggregates, before the sample injection, capillary was rinsed for 10 min at 90 kPa with the micelle dispersed in the BGE which was also used as the background electrolyte. Dipeptide samples were introduced into the capillary tube at concentration of 5.0 mM. After each run the capillary was rinsed for 1.0 min at 90 kPa in order to refresh the electrolyte solution filled in the capillary tube. Methanol and Phenanthrene were used as markers of the electro-osmotic mobility and of the electrophoretic mobility of the micelles respectively.

[11] Corradini, D.; Cogliandro, E.; D'Alessandro, L.; Nicoletti, I. *J. Chromatogr. A* **2003**, *1013*, 221-232.

### *5.5.2 Chemicals and Samples*

Sodium *N*-dodecanoyl-L-prolinate was prepared according to literature procedures for the formation of amides of amino acids.[12] The product was crystallised from n-butyl ether. Reagent-grade phosphoric acid, hydrochloric acid, sodium hydroxide, sodium chloride were purchased from Carlo Erba (Milan, Italy). Deionized water was obtained by a Milli-Q water purification system from Millipore (Bedford, MA, USA) and degassed by sonication before use. Mesityl Oxide and Phenanthrene were obtained by Fluka (Milan, Italy). LL/DD enantiomeric couple of ditryptophan were purchased from Research Plus Inc (Manasquan, NJ, USA). L-proline were obtained from Fluka (Milan, Italy). LD and DL-Trp were synthesized according to literature procedures.[5]

### *5.5.3 Buffers*

Buffers consisting of phosphoric acid and its sodium salt were prepared by titrating the proper amount of requested acid to the desired pH value with 1.0 M sodium hydroxide and by adding the desired amount of sodium chloride. The pH was measured with a glass electrode Model 52–02 and Model Basic 20 pH-Meter, both from Crison (Alella, Spain). All solutions were filtered through a type HA 0.22μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sonication before use.

[12] Jungermann, E.; Gerecht, J.F.; Krems, I.J. *J. Am. Chem. Soc.* **1956**, *78*, 172-174.