Application of Protein–Liposome Conjugate as a Pseudo-Stationary Phase in Capillary Electrophoresis

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Abstract

Liposomes have very similar structure to cell plasma membranes. Using liposomes as stationary phase in liquid chromatography (LC) or micellar electrokinetic chromatography (MEKC) has been demonstrated to be a good, dynamic method for the study of the interaction between cell membranes and important biomolecules. There has been no report on integrating plasma membrane proteins with phospholipids as pseudo-stationary phase in MEKC. In this paper, a novel mode of capillary electrophoresis (CE) is developed, that is, protein–liposome conjugate. This protein–liposome biomimetic membrane is demonstrated for the first time to be applicable as pseudo-stationary phase in MEKC. The protein is able to significantly improve chromatographic performance and stability. The experimental phenomena are further confirmed in terms of specific capacity factors and free binding energy. This new CE mode is used to investigate the interaction between dopamine transporter and dopamine–nomifensine.

Introduction

Liposomes are self-assembled vesicles commonly consisting of phospholipid bilayers, which have the amphiphilic character of the phospholipid property of encapsulating hydrophobic compounds in the bilayer membrane, or hydrophilic molecules in the internal cavity (1). In addition, depending on phospholipid composition, size, and surface characteristics, liposomes can establish a variety of interactions with molecular species and cell surfaces in the surrounding solution (2).

These features enable liposomes to be widely employed as models for biological membranes as well as carriers for drugs and other agents of therapeutic, diagnostic, and cosmetic value (3). The use of phospholipid coating as a stationary phase in liquid chromatography has given rise to the study of solute membrane interactions and, to a lesser extent, in achieving several solute separations (4).

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Capillary electrophoresis (CE) has a great deal of characteristics: (*i*) requiring a small amount of liposomes and samples, which is especially appealing when dealing with membrane transport proteins which are difficult to extract and with limited amount; (*ii*) being convenient and flexible in applying different modes, which are beneficial for complicated analysis; (*iii*) ability to employ liposomes not only coating as stationary phase, but also acting as a pseudo-stationary phase in a simple manner without the need of immobilization, that might destroy biological native structure; (*iv*) owning potential of constructing a high throughput and highly efficient screening platform for the evaluation of drug–membrane permeation ability or protein interaction.

Liposomes have recently been found to have a variety of applications in CE, involving their use as coating materials (5–8) or carriers (9–13) to achieve solute separation or to investigate solute membrane interactions. Hjerten et al. were the first to use liposomes in CE for studying their interactions with model drugs and two octapeptides (14). Liposome–water partition coefficients for drugs were determined (12–13). Interactions with and transport of small molecules through cell membranes were simulated and studied using CE techniques (15–18).

Liposomes have been applied for protein analysis, the separation of proteins (1), protein biopartition, and bioaffinity (19–23). However, to date, there is no such report that proteins are employed as one of the components in liposome microemulsion for micellar electrokinetic chromatography (MEKC) analysis. In this study, it is proposed that protein–liposome conjugate could not only be a good mode for the separation, but also for the investigation of protein interaction.

The dopamine transporter (DAT) is a plasma membrane protein expressed exclusively in DA synthesizing neurons. It plays a crucial role in dopaminergic neurotransmission by taking up extracellular DA into nerve cells, terminating DA neurotransmission, and in maintaining DA homeostasis in the central nervous system (24–26). DAT has been taken as the molecular target for therapeutic agents used in the treatment of mental disorders (27). The mechanism that regulates DA uptake is of medicinal importance as potential sites of action for Parkinson's and other neurological diseases, and also for psychiatric diseases (28).

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In this article, DAT-based liposome conjugate was constructed for the new CE mode. In order to substantiate one of its potential applications in the drug screening, interaction between DAT and DA as well as nomifensine, a specific inhibitor for DAT, was investigated as an example.

Experimental

Materials

DA, nomifensine (1,2,3,4-tetrahydro-2-methyl-4-phenyl-8-isoquinolinamine), Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO). Egg phosphatidylcholine was obtained from Beijing Shuangxuan Microorganism Substrates Factory (Beijing, China). Cholesterol and other chemicals were all of analytical grade and purchased from Beijing Chemical Reagents Company (Beijing, China). Phosphate-buffered saline (NaH_2PO_4–Na_2HPO_4, PBS, 10mM, pH 6.8) that was close to the physiological environment of human body was chosen as the background electrolyte (BGE) solution in MEKC. All aqueous solutions were prepared using water purified with a Milli-Q purifier system (Millipore, Milford, MA).

Preparation of protein

The SH-SY5Y cells were the resource for plasma membrane protein preparation. Cells in culture medium of 90% (v/v) DMEM and 10% (v/v) FBS were cultured in a CO_2 incubator (Sanyo Electric Co., Osaka, Japan). Culture medium was removed from the cells, which were then washed with cold phosphate buffered saline (PBS). Cells were detached from culture flasks with cold lysis buffer and centrifuged for 20 min at 4°C at 1,000 × *g*. Cold lysis buffer was removed and cells were then resuspended in ice-cold PBS. The cells were broken up by ultrasonic lysis. After that, the admixture obtained was centrifuged for 30 min at 4°C at 800 × *g*. The supernatant was centrifuged again for 30 min at 4°C at 17,000 × *g*. The supernatant was collected. An experiment of sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was carried out to verify the successful preparation of DAT from SH-SY5Y cells. SDS–PAGE was performed on 10% polyacrylamide. Gels were stained for 60 min with 0.1% (w/v) Coomassie Brilliant Blue R-250 in methanol–glacial acetic acid–water (40:10:50, v/v/v), then destained for 5 h in methanol–glacial acetic acid–water (10:10:80, v/v/v). The final protein stock solution was stored at –80°C for use.

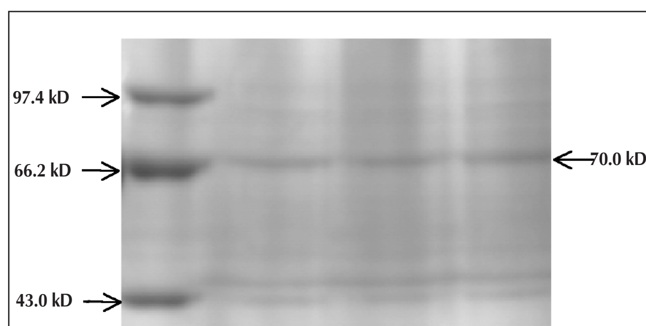

Figure 1. The SDS–PAGE analysis of DAT. SDS–PAGE was performed on 10% polyacrylamide. Gels were stained for 60 min with 0.1% (w/v) Coomassie Brilliant Blue R-250 in methanol–glacial acetic acid–water (40:10:50, v/v/v), then destained for 5 h in methanol–glacial acetic acid–water (10:10:80, v/v/v).

Preparation of liposome buffer (12)

160 mg of egg phosphatidylcholine and 53 mg of cholesterol were weighed out and transferred to a 50-mL round-bottom flask. Then, 20 mL chloroform was used to dissolve the egg phosphatidylcholine and cholesterol. The round-bottom flask was placed on a rotary evaporator at a temperature of 40°C and allowed to rotate at the moderate rotation rate for 20 min. This was done in order to ensure complete dissolution of the egg phosphatidylcholine and cholesterol in the organic solvent. At the same time, vacuum was applied, and the egg phosphatidylcholine cholesterol mixture was allowed to dry and adhere to the glass surface. An additional 10 min drying time was included in the procedure to ensure that no trace amounts of organic solvent were present. After the drying process was complete, 8 mL of Milli-Q water was added to the dry lipid layer, the flask was swirled around to form multilamellar vesicles (MLV), and then ultrasonication was performed for 3 h at room temperature to produce a liposome suspension. The final product was a relatively clear homogeneous solution of small unilamellar vesicles (SUV). The liposome suspension was frozen at –37°C for 24 h and then freeze-dried until yellow powder formed. The required amount of freeze-dried liposomes were added to BGE solution (NaH_2PO_4–Na_2HPO_4, 10mM, pH 6.8), and then ultrasonicated to make liposome solution. All liposome solutions were used on the day they were prepared.

Preparation of protein–liposome conjugate

A certain volume of DAT solution was added into the liposome solution. The mixture was blended for immobilization on a shaker in an ice-bath environment for 3 h. The mixture was then centrifuged for 30 min at 4°C at 17,000 × *g*. The deposition obtained was used as pseudo-stationary phase in protein–liposome CE mode. By subtracting the amount of protein left in the supernatant from the overall protein amount initially input, the immobilization efficiency of protein into liposome was calculated.

The protein concentration was measured by the Bradford method. A PBS buffer containing the protein–liposome conjugates was prepared by dissolving a certain amount of protein–liposome conjugates into PBS.

Electrophoretic analysis

MEKC separation was performed with Unimicro TriSep-2100GV pressure-loading capillary electrochromatography system equipped with chromatography workstation (Unimicro Technologies, Pleasanton, CA). All the experiments were run at an applied voltage of 10.0 kV. Analytes were detected by a UV–vis detector at 254 nm. A bare fused-silica capillary (50-µm i.d., 375-µm o.d., Yongnian Optical Fiber Factory, Hebei, China) was used for analysis. Its total length was 80 cm, and the effective length

was 50 cm. Before use, the new capillaries were flushed with 1 mol/L NaOH, 0.1 mol/L NaOH, and pure water for 1 h once, successively. The detection window (0.5 cm) on the capillary was made by removing the polyimide coating outside.

The analytes were DA and nomifensine. Three kinds of electrophoresis modes using different electrophoresis mediums were studied and compared. The three mediums were pure PBS buffer, liposome buffer, and protein–liposome conjugate buffer, respectively.

Results and Discussion

The immobilization of proteins in liposomes

The experiment of SDS–PAGE was applied to investigate the existence of DAT. The first lane in the SDS–PAGE graph (Figure 1) was markers of 97.4, 66.2, and 43.0 kD. The following three lanes were applied with the same protein sample prepared. The presence of 70.0 kD DAT in three parallel analysis demonstrated our successful identification of DAT from SH-SY5Y cells.

The concentration of protein stock solution measured by the Bradford method was 0.135 mg/mL. After immobilization into liposome (0.3 mg/mL), the concentration of the protein in supernatant was 0.007 mg/mL. The immobilization efficiency of 93.1% displayed that the immobilization of protein into liposome was achieved.

The electropherograms of DA and nomifensine in three kinds of CE modes are shown in Figure 2. Some interesting phenomena were revealed in the studies of analytes binding with pseudo-stationary phase in electrophoresis analysis. After changing the CE mode using PBS buffer to liposome buffer, the baseline of electropherogram (Figure 2B) became worse with the injection of analytes. Because the hydrophobic nature of the bilayer of liposomes favored the embedment of nomifensine and DA, the stable stationary originally formed might be disturbed in the presence of nomifensine and DA. This resulted in a poor signal-to-noise ratio. In protein–liposome mode, the decreased relative standard deviation (RSD) value of migration time and the smoother baseline strongly demonstrated that the stability of the system was improved (Figure 2C). This phenomenon displayed that the protein–liposome conjugate existed in stable state. In other words, DAT was found to be able to stabilize liposome vesicles. This finding was different with the report that liposomes became destabilized in the presence of serum (29). It should also be noted that, even in DAT–liposome mode, the baseline still became worse after the injection of nomifensine exceeded a certain limit (data not shown). It might be due to the reason that too much nomifensine bound to DAT and liposomes, changing the steric structure and crashing the membrane. The decreased stability of DAT–liposome conjugate resulted in baseline deformation. Thus, it was proposed that there existed equilibrium when a stable protein–liposome complex came into being and functioned as a whole. The baseline deformation caused by nomifensine after a certain stable stage revealed that DAT well retained its ability to bind nomifensine.

Interaction between DA/nomifensine and protein–liposome conjugate

As shown in Figure 2A, nomifensine migrated behind DA in PBS CE mode because of its different size and electric charge. In the CE mode with liposome buffer, liposomes typically resulted in the delay of migration for both analytes (Figure 2B). The hydrophobic interaction between analytes and liposomes contributed to decreased migration velocity. In the DAT–liposome mode, the migration time for both DA and nomifensine was even longer than that in liposome mode (Figure 2C). DAT immobilized instead of viscosity was apparently shown to play a larger role than liposome in this remarkable analyte migration retardation. It corresponded well with the fact that both DA and nomifensine had specific interaction with DAT.

The different extent of migration change for DA and nomifensine was also clearly shown in Figure 3. The migration time of DA in liposome buffer was 2.4 min more than in PBS buffer, while it was nearly 5 min prolonged for nomifensine. Generally, an increase in migration can usually be ascribed to factors such as the electroendosmosis flow, viscosity, or steric change of the running buffer besides interactions of analytes with stationary phase. Comparing with a liposome vesicle whose diameter fell into nanometer range, the tiny size of DA and nomifensine made their own size difference negligible. That meant the viscosity introduced by liposomes produced similar influences to both analytes. Upon that, in this

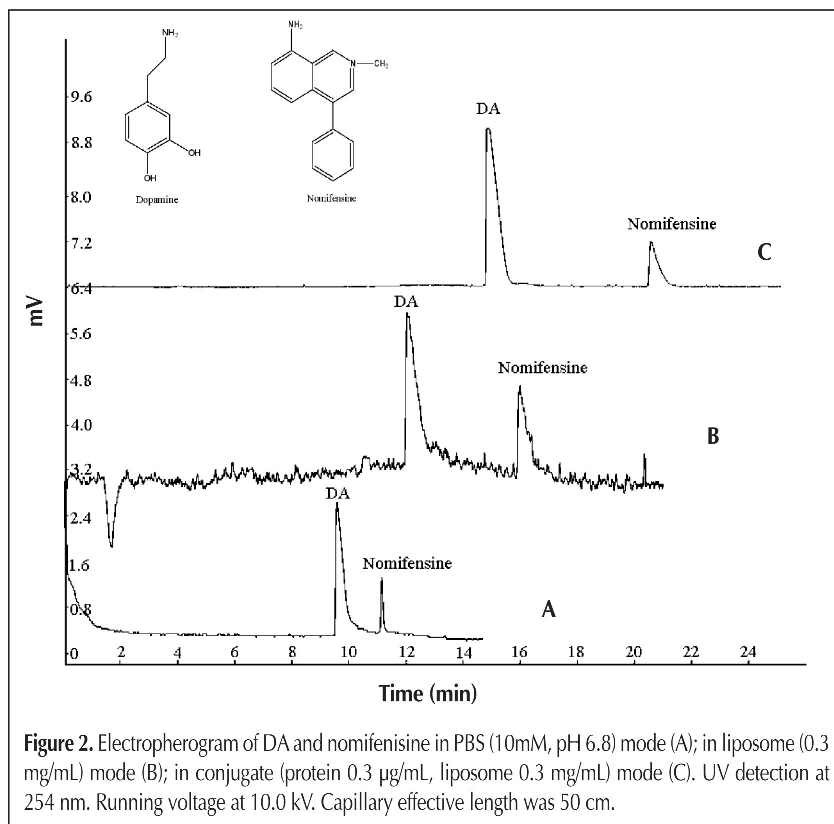

Figure 2. Electropherogram of DA and nomifenisine in PBS (10mM, pH 6.8) mode (A); in liposome (0.3 mg/mL) mode (B); in conjugate (protein 0.3 µg/mL, liposome 0.3 mg/mL) mode (C). UV detection at 254 nm. Running voltage at 10.0 kV. Capillary effective length was 50 cm.

experiment, the distinctive migration delay of nomifensine demonstrated that nomifensine had stronger interactions with liposomes than DA. It was in accordance with the fact that hydrophobic quinoline and aromatic ring structure had advantages in penetrating nomifensine molecules into the bilayer of liposomes. The similarity was held true in DAT–liposome mode as in liposome mode. The different migration of DA and nomifensine was investigated in detail. The prolonged migration time of nomifensine was almost twice as that of DA. Because different viscosity influences brought by DAT–liposome conjugates could also be neglected, experimental observation gave such elucidation that nomifensine had stronger interactions with DAT–liposome than DA. It is in agreement with the fact that nomifensine, being a competitive DAT inhibitor (30), has stronger ability to occupy the binding sites of DAT, which reduced the binding of DA to DAT. Otherwise, the postponement of migration for DA and noncompetitive nomifensine would be the same in the presence of DAT. So, through the binding, longer retardation, and successful release from DAT–liposome, this CE mode adequately exhibited the reversible and competitive kinetic mechanism of nomifensine inhibition in an easy manner. On the other hand, because the migration separation behavior of analytes completely reflected and corresponded to the strength of the bioaffinity, it was concluded that DAT was successfully obtained from SH-SY5Y cells and DAT contained in the conjugate satisfactorily retained its biological discrimination activity during the process of CE.

Good repeatability in DAT–liposome mode was demonstrated by its low value of the RSD data obtained for five measurements, as shown in Table I. For any of the three modes, the column efficiency, as high as 10^6 effective plates/m, exhibited good performance of this system, especially for DAT–liposome mode.

Calculation of specific capacity factor *K*s and $\Delta(\Delta G^0)$ of DA and nomifenisine

In accordance with the study by Zhang et al. (14), the specific capacity *K*s was calculated to obtain a normalized retention quantity.

$$Ks = k'/B = (t - t')/(t' \times B) \qquad \text{Eq. 1}$$

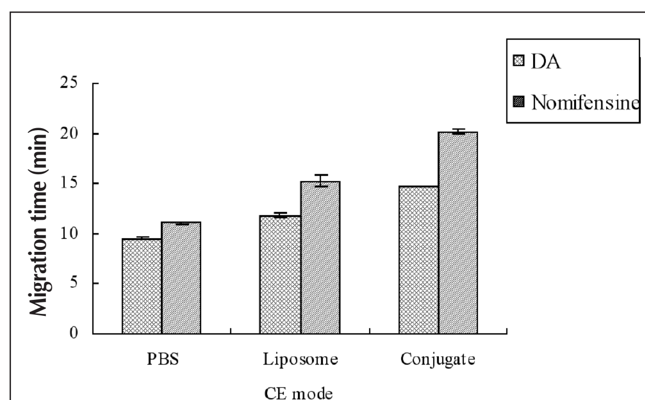

Figure 3. Comparison of migration time of DA and nomifensine in different kinds of CE modes.

With k' as the capacity factor; B, the concentration of liposome in the running buffer; t, the electrophoretic migration time of an analyte in the presence of liposomes (or DAT–liposome); and t', the electrophoretic migration time of an analyte in the absence of liposomes (or DAT–liposome). The order of retardation caused by sample partitioning between the liposomes and the free buffer (*i*) was nomifensine > DA; the order of retardation caused by sample partitioning between the conjugate and the free buffer (*ii*) was nomifensine > DA; and the order of retardation caused by sample partitioning between the conjugate and the liposomes (*iii*) was also nomifensine > DA (Table II). The different value of specific capacity factor *K*s is again consistent with the fact that nomifensine has stronger interaction with both liposome and DAT-liposome conjugate than DA.

The $\Delta(\Delta G^0)$ values for nomifensine relative to DA in different mediums were calculated as follows:

$$\Delta(\Delta G^0) = \Delta G^0_2 - \Delta G^0_1 = -R_g T \ln[(t_2/t_2' - 1)/(t_1/t_1' - 1)] \qquad \text{Eq. 2}$$

where $\Delta G^0 = -R_g T \ln[(t/t' - 1)/\phi]$; subscript 1 and 2, DA and nomifensine, respectively; t, the electrophoretic migration time of an analyte in the presence of liposomes (or DAT-liposome); t', the electrophoretic migration time of an analyte in the absence of liposomes (or DAT–liposome); gas constant R_g = 8.314 J/deg/mol; and the internal temperature of the capillary $T \approx 295$ K. The $\Delta(\Delta G^0)$ value between nomifensine and DA in liposome mode and PBS mode (*i*) was –1.229 kJ/mol. The $\Delta(\Delta G^0)$ value between nomifensine and DA in conjugate mode and PBS mode (*ii*) was –1.036 kJ/mol. The $\Delta(\Delta G^0)$ value in conjugate mode and liposome mode (*iii*) was –0.602 kJ/mol (shown in Table II).

Taking DA as reference standard, the negative values of $\Delta(\Delta G^0)$ of nomifensine displayed nomifensine's higher tendency to bind with the stationary phase. These results were in line with the fact that the inhibitor had a higher affinity with the conjugate than with liposome. The decreased difference between the ΔG^0 value of DA and nomifensine on changing to DAT–liposome mode, illustrated that the stronger interaction of both analytes with protein diminished their disparity brought by other interactions. Because the accuracy of the ΔG^0 value depends on the detailed knowledge of phase ratio, which is difficult to estimate, the value

Table I. The Electrophoretic Data Comparison of DA and Nomifensine in the Three Kinds of CE Modes

Peak	Average migration time (min)	RSD (%) of migration time (n = 5)	Column efficiency (effective plates/m)
DA	9.554*	0.41*	101084*
	11.755†	1.33†	202420†
	14.705‡	0.36‡	753312‡
Nomifensine	11.067*	0.83*	131546*
	15.274†	3.28†	181828†
	20.172‡	1.47‡	575274‡

* PBS mode.
† liposome mode.
‡ conjugate mode.

Table II. The Value of Specific Capacity Factor Ks and Ø(ØG0) of DA and Nomifensine*

Analyte	(*i*)		(*ii*)		(*iii*)	
	Ks^{i} (mL/mg)	$Ø(ØG^{0})^{i}$ (kJ/mol)	Ks^{ii} (mL/mg)	$Ø(ØG^{0})^{ii}$ (kJ/mol)	Ks^{iii} (mL/mg)	$Ø(ØG^{0})^{iii}$ (kJ/mol)
DA[1]	0.768	–1.229	1.797	–1.036	0.837	–0.602
Nomifensine[2]	1.267		2.742		1.069	

* (*i*) denoting comparison of liposome mode with PBS mode; (*ii*) denoting comparison of conjugate mode with PBS mode; (*iii*) denoting comparison of conjugate mode with liposome mode.

of $\Delta(\Delta G^0)_{iii}$ could not be deduced from value of $\Delta(\Delta G^0)_{i}$ and $\Delta(\Delta G^0)_{ii}$. It might give the hint that the stably existing protein–liposome conjugate might interact with guests synergistically as a whole, not individually. Therefore, the semiquantitative $\Delta(\Delta G^0)$ information obtained here made possible the studies of membrane interactions with the help of CE techniques. Further and systematic study on the interactions between DAT and DA is ongoing in our lab.

Conclusion

Our new MEKC model displayed the feasibility that the protein–liposome biomimetic conjugates could be used as pseudo-stationary phase in CE to make membrane investigations. Because liposomes have very similar structure to cell plasma membranes, into which plasma membrane proteins were immobilized to produce the protein–liposome conjugate, the conjugate can be regarded as mimic cell. The innovation of the present method is to apply this kind of mimic cell into CE. However, this paper is only a preliminary study on the feasibility of this novel method. Further study on the biological activity of conjugate in MEKC is ongoing in our lab. But there is no doubt that this CE mode holds great potential in the field of interaction investigation, separation, drug screening, the field of proteomics, and so on.

Acknowledgments

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