ELSEVIER

Journal of Chromatography A, 813 (1998) 402–407

JOURNAL OF CHROMATOGRAPHY A

Short communication

Migration behavior of dyestuff-containing liposomes in capillary electrophoresis with chemiluminescence detection

Kazuhiko Tsukagoshi*, Yasuo Okumura, Riichiro Nakajima

Department of Chemical Engineering and Materials Science, Faculty of Engineering, Doshisha University, Kyotanabe, Kyoto 610-03, Japan

Received 10 June 1997; received in revised form 27 April 1998; accepted 28 April 1998

Abstract

Various types of dyestuff-containing liposomes were prepared from eosin Y or rhodaminine B, dipalmitoylphosphatidylcholine or dipalmitoylphosphatidylcholine–dipalmitoylphosphatidylserine mixtures, and buffer solutions (trapped solutions) differing in chemical species, buffer concentrations, and pH. The dyestuff-containing liposomes were subjected to capillary electrophoresis with chemiluminescence detection, and the effects of the constituents on liposome properties were examined from the obtained electropherogram. Two peaks were typically recorded on the electropherograms; one was due to dyestuff entrapped in the liposomes and another was due to free dyestuff in the bulk solution. The changes of retention times and ratios of the two peaks easily and rapidly offered useful information as to permeability and surface charge of the liposome membranes.

Keywords: Liposomes; Eosin Y; Rhodamine B; Dyes; Dipalmitoylphosphatidylcholine; Dipalmitoylphosphatidylserine; Lipids

1. Introduction

In the past decade, capillary electrophoresis (CE) has become one of the most powerful and conceptually simple separation techniques for the analysis of complex mixtures. CE features high resolution, relatively short analysis times, and low operational cost compared to high-performance liquid chromatography. Furthermore, when combined with CE, chemiluminescence (CL) can offer excellent analytical sensitivity and selectivity. We have reported on CE with CL detection for the separation and determination of small amounts of metal ions, metal complexes, dyestuffs, proteins, and alkaloids. [1–3]

Recently, the behavior of liposomes in CE was examined for characterizing liposomes prepared for use in industrial and analytical applications. [4] Liposomes were also used as a pseudostationary phase for the analysis of drugs by CE. [5] In our previous papers, [6,7] dyestuff-containing liposomes prepared from eosin Y and dipalmitoylphosphatidylcholine (DPPC) were for the first time analyzed by CE–CL using the peroxyoxalate system. The following matters were examined by the use of the electropherograms: the difference between multilamellar vesicles and small unilamellar vesicles (SUVs), the liposome preparation process including gel filtration and ultrasonic irradiation, and the effect of standing time. Only CL detection was effective for examining the electrophoretic behavior of the

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00376-8

eosin Y-containing liposome in a capillary. Spectrophotometric and fluorescent detection could not be utilized for detecting the liposome, mainly due to the low sensitivities.

In the present study, various types of dyestuff-containing liposomes were prepared and analyzed by CE–CL. It was found that the constituents of the liposome, which were dyestuff, lipid, and trapped solution, influenced the properties of the liposome and hence affected their migration behavior. The present CE–CL system will be useful for characterizing liposomes which have been widely used as models for biomembranes, as drug carriers, and for other purposes.

2. Experimental

2.1. Reagents

All reagents used were of commercially available and special grade. Ion-exchanged water was distilled before use. Dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylserine (DPPS) were purchased from Sigma and Funakoshi, respectively. Bis(2,4,6-trichlorophenyl)oxalate (TCPO) was obtained from Wako. Eosin Y and rhodamine B were purchased from Nacalai Tesque.

2.2. Preparation of dyestuff-containing liposome

Standard eosin Y-containing liposomes were prepared as follows. DPPC (2 mg) was dissolved in chloroform (3 cm^3) in a 10-cm^3 round-bottomed flask and dried on a rotary evaporator under reduced pressure at 37°C to form a thin film on the flask wall. An eosin Y solution of 10 m*M* in a 10 m*M* sodium carbonate buffer solution (pH 9.0) was added to the film. The mixture was treated at 55°C for 2 h by ultrasonic irradiation. The obtained suspension was subjected to gel filtration (Sephadex G-50, Nick column, bed volume 2 cm×1 cm I.D.; Pharmacia Biotech) using 10 m*M* sodium carbonate (pH 9.0) as an eluent in order to remove the free eosin Y.

Various types of dyestuff-containing liposomes were prepared in the same manner as the above-mentioned standard eosin Y-containing liposome, but with different constituents (dyestuff, lipid, and trapped solution). Lipid content ratio was as follows, DPPC(mg):DPPS(mg)=2.0:0.05, 2.0:0.1, or 2.0:0.2. Dyestuff was 0.1, 1.0, 10, or 100 m*M* eosin Y or 10 m*M* rhodamine B. Buffer solution as trapped solution in liposome interior was 0.5, 1.0, 10, or 100 m*M* sodium carbonate (pH 9.0) or 10 m*M* phosphate buffer (pH 7.0, 9.0, or 11.0). Liposomes not containing dyestuff were also prepared in the same manner as the standard eosin Y-containing liposomes.

2.3. Apparatus and procedure

Liposomes were observed by means of transmission electron microscopy (TEM) (Hitachi-H8100). A CE–CL system similar to that reported in a previous paper [1] was used for the present study. A fresh fused-silica capillary tube (70 cm×50 μm I.D.) was treated with 1 m*M* sodium hydroxide for 30 min and was then washed with distilled water. A migration buffer solution (10 m*M* sodium carbonate; pH 9.0) was filtered and degassed. The capillary tube was filled with the buffer solution. The sample solution was introduced at the positive electrode side for 15 s from a height of 30 cm by siphoning. A voltage of 15 kV was applied to the electrodes using a d.c. power supplier (Model HCZe-30PNO.25, Matsusada Precision Devices). A 500 μm I.D. PTFE tube, which was covered with black Tygon-tape and had a 8 mm detection length in front of a photomultiplier (Hamamatsu Photonics R-464), was used as a detection cell (Fig. 1). A CL reagent solution (50 cm^3

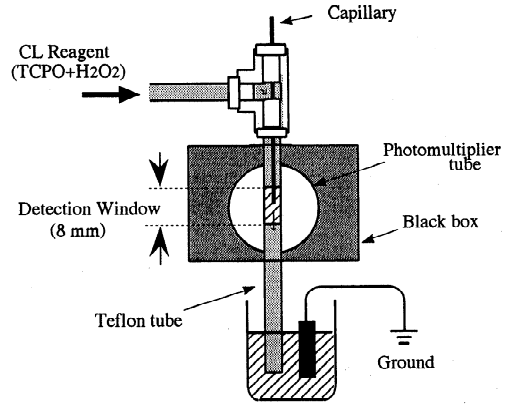

Fig. 1. Schematic diagram of CL detector.

of 1 m*M* TCPO acetonitrile solution+288 μl of 30%, w/w, aqueous solution of H_2O_2) was fed at a rate of 30 μl min^{-1} by a pump (Tosoh CCPD type) to be mixed with the eluate at the tip of the capillary tube. The CL detected by the photomultiplier was measured by a photon counter (Hamamastu Photonics C1230) with an integrator (Shimadzu Chromatopac C-R6A).

3. Results and discussion

3.1. Characterization of liposome as SUV

In this study, liposomes were prepared as SUVs according to the ordinary method [8,9]. Lipid concentrations in the suspensions were estimated to be about 2.7 m*M* and 0.3 m*M*, corresponding to those before and after gel filtration, respectively. Liposomes not containing eosin Y were examined as follows, in order to confirm the formation of SUVs. Trapping efficiency, which is generally expressed as a percentage of the amount of dyestuff entrapped in liposome to the initially added amount, was estimated for calcein-containing liposomes. The obtained value of 3.43% seems to be slightly large, and is almost in agreement with that of 3.55% for SUVs which were previously prepared [10] by reversed-phase evaporation method. The liposome size was measured by means of TEM, which confirmed that the liposomes had submicron dimensions as SUVs [9,11] and assumed a spherical shape. However, TEM observation indicated a somewhat broad distribution. The small number of large size SUVs may contribute to the above comparatively large trapping efficiency.

3.2. Effect of eosin Y concentration in the preparation on CL intensity

In Fig. 2 the relationship between eosin Y concentration used for preparation of the liposomes and CL intensities obtained by the CE–CL system is shown. The CL intensities, both peak height and peak area, increased with increasing concentrations of eosin Y. However, at the high concentration of 100 m*M* eosin Y, the liposomes could not be prepared with good reproducibility. Therefore, the 10 m*M* eosin Y concentration was selected as standard in the present work for the preparation of liposomes.

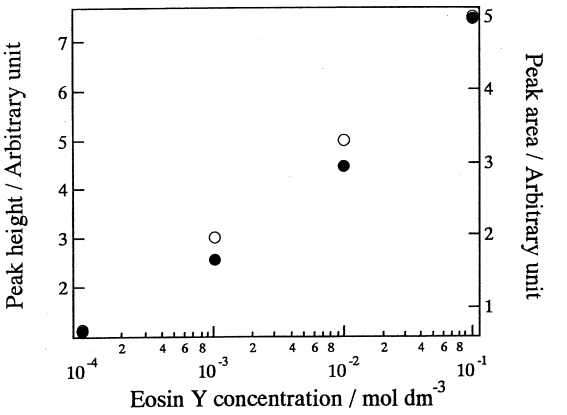

Fig. 2. Relationship between eosin Y concentrations in the liposome preparation and CL intensities. (○) peak height; (●) peak area. Conditions: capillary, 70 cm×50 μm I.D. fused silica; applied voltage, 15 kV; migration buffer, 10 m*M* sodium carbonate buffer (pH 9.0); CL reagent, a mixture of 1 m*M* TCPO acetonitrile solution of 50 cm^3 and 30% (w/w) H_2O_2 aqueous solution of 288 μl; and liposome preparation; 2 mg DPPC in 3 cm^3 chloroform, 10 m*M* sodium carbonate buffer (pH 9.0) as trapped solution.

3.3. Comparison of electropherograms for eosin Y- and rhodamine B-containing liposomes

Although eosin Y and rhodamine B are common dyestuffs for the TCPO–H_2O_2 CL, the former dye gave about ten times greater sensitivity than did the latter one in this CE–CL system. Eosin Y or rhodamine B-containing liposomes were prepared using a dye concentration of 10 m*M*. Free rhodamine B could not be removed sufficiently through gel filtration. The obtained electropherograms were compared with each other, together with the molecular structure of the dyestuffs (Fig. 3). Free eosin Y appeared after the entrapped eosin Y in the liposomes, while free rhodamine B appeared before the entrapped rhodamine B. It was reasonable to consider the charge properties of these dyestuffs in alkaline condition. In both electropherograms liposomes which entrapped the dyestuffs were detected at almost the same migration time of 9 min. This means that the charge of the dyestuff entrapped in

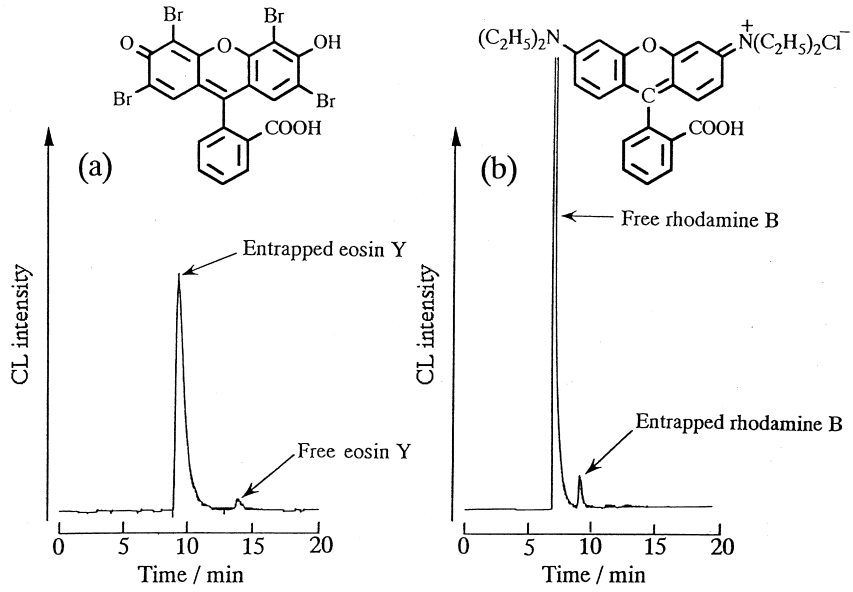

Fig. 3. Electropherograms of (a) eosin Y- and (b) rhodamine B-containing liposomes as well as molecular structures of the dyestuffs. The experiments were carried out under the same conditions as described in Fig. 2, except for liposome preparation; 10 m*M* eosin Y or rhodamine B, 2 mg DPPC in 3 cm^3 chloroform, 10 m*M* sodium carbonate buffer (pH 9.0) as trapped solution.

the liposomes did not influence the migration of the liposomes.

3.4. Effect of the lipid content ratio of the liposomes on the electropherogram

Various ratios of DPPC and DPPS were used in the preparation of eosin Y-containing liposomes. The obtained electropherograms are shown in Fig. 4. The peak pattern in the electropherogram for the DPPC–DPPS liposomes was similar to that of the DPPC liposomes. However, the first peak due to liposomes or eosin Y in the liposomes was recorded later with increasing content of DPPS. In the third panel, the liposome peaks shifted such that they were on top of the eosin Y peak causing peak distortion. DPPC has a quaternary amine moiety in the molecule, while DPPS has serine as the amphoteric moiety. Therefore, the DPPC–DPPS liposomes must be more negatively charged than the DPPC liposomes are under the present conditions. The negative charge would cause a delay in the migration time of the liposome.

The results support the possibility that an observation of migration behavior of dyestuff-containing liposome by CE–CL provides information concerning the membrane composition and surface charge of the liposomes.

3.5. Effect of trapped solution on stability or permeability of liposome membrane

The eosin Y-containing liposomes were prepared using various buffer solutions as trapped solutions. They were then left in a 10 m*M* sodium carbonate buffer (pH 9.0), the same as the CE migration buffer. The effects of the anion species (carbonate and phosphate ion), the buffer concentrations, and the pH of the solutions trapped in the liposome interior on electropherograms were examined as a function of standing time. The ordinate is expressed as peak area ratio of free to entrapped eosin Y. An increase in the ratio indicates a degree of release of eosin Y from the liposome interior to the bulk solution, that is, instability or permeability of the liposome membrane. The anion species and the pH did not greatly influence the instability or permeability of the liposomes under the present experimental conditions (the data is not shown); the ratio of peak areas gradually increased with increasing standing time, similarly as

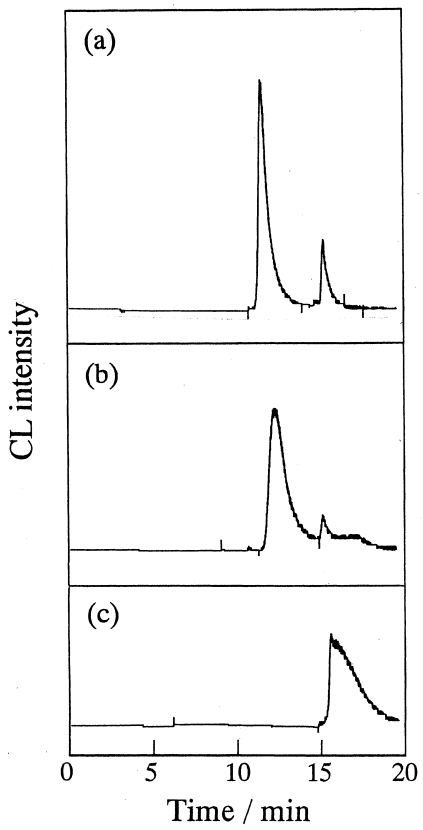

Fig. 4. Effect of DPPC:DPPS content ratio of liposomes on the electropherogram. DPPC (mg):DPPS(mg)=(a) 2.0:0.05, (b) 2.0:0.1, and (c) 2.0:0.2. The experiments were carried out under the same conditions as described in Fig. 2, except for liposome preparation; 10 m*M* eosin Y, DPPC–DPPS mixture in 3 cm^3 chloroform, 10 m*M* sodium carbonate buffer (pH 9.0) as trapped solution.

for the standard sample of liposomes which was prepared using a 10 m*M* sodium carbonate buffer (pH 9.0).

On the other hand, the buffer concentrations in the liposome interior influenced the instability or permeability of the liposome membrane to a great extent (Fig. 5). The liposomes which included lower sodium carbonate concentration (0.1 and 1.0 m*M*) than that of bulk solution (10 m*M*) showed higher peak area ratios than the standard liposomes did, which included the same sodium carbonate concentration as that of bulk solution. In contrast, the liposome which included higher carbonate concentration (100 m*M*) showed no change in the ratio within 48 h standing

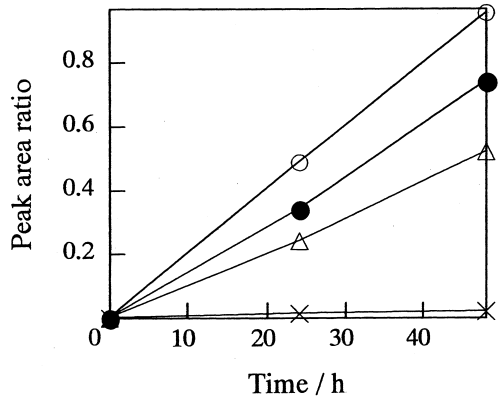

Fig. 5. Effects of buffer concentrations of trapped solution on peak area ratio. (○) 0.1 m*M* sodium carbonate (pH 9.0), (●) 1.0 m*M* sodium carbonate (pH 9.0), (△) 10 m*M* sodium carbonate (pH 9.0), and (×) 100 m*M* sodium carbonate (pH 9.0). The ordinate is expressed as peak area ratios of free eosin Y to entrapped eosin Y. The experiments were carried out under the same conditions as described in Fig. 2, except for liposome preparation; 10 m*M* eosin Y, 2 mg DPPC in 3 cm^3 chloroform, various buffers as trapped solution.

time. That is, the lower concentration of buffer accelerated a release of eosin Y and a higher concentration suppressed it. This reflects an osmotic effect of the liposome membrane [12]. If the interior concentration of some chemical species is lower than the exterior one, internal water moves to the outside in order to keep an osmotic balance. The liposome, consequently, contracts (an osmotic shrinkage). In contrast, if the inside concentration is higher, the liposome swells (an osmotic swelling). The release behavior of eosin Y which was indicated as the effect of buffer concentrations in Fig. 5 may be caused by the osmotic shrinkage.

Dyestuff-containing liposomes, which are also employed in immunoassay, [13,14] have been utilized for characterizing liposomes (homogeneity, trapped volume, stability, permeability, etc.). [10,15,16]. The dyestuff entrapped in liposomes plays an important role as a probe (what is called ‘a release marker’). However, the methods for measuring the probe have necessitated tedious treatments, such as centrifugation, gel filtration and dialysis, and, in general, have provided only indirect information. The results of this report clearly showed that the CE–CL is not only effective for the characterization

of liposomes, but also promising as a means of separation and detection for a release marker.

Acknowledgements

We wish to thank Mr. A. Arai of Shimazu Corporation for his kind discussions. We would also like to thank Dr. S. Aono at Doshisha University for measuring TEM. This work was in part supported by a grant to RCAST at Doshisha University from the Ministry of Education, Japan and by a grant-in-aid for Scientific Research from the Ministry of Educations, Science, Sports and Culture, Japan.

References

[1] K. Tsukagoshi, A. Tanaka, R. Nakajima, T. Hara, Anal. Sci. 12 (1996) 525.
[2] K. Tsukagoshi, S. Fujimura, R. Nakajima, Anal. Sci. 13 (1997) 279.
[3] K. Tsukagoshi, K. Miyamoto, E. Saiko, R. Nakajima, T. Hara, K. Fujinaga, Anal. Sci. 13 (1997) 639.
[4] M.A. Roberts, L. Locascio-Brown, W.A. MacCrehan, R.A. Durst, Anal. Chem. 68 (1996) 3434.
[5] Y. Zhang, R. Zhang, S. Hjertén, P. Lundahl, Electrophoresis 16 (1995) 1519.
[6] K. Tsukagoshi, H. Akasaka, R. Nakajima, T. Hara, Chem. Lett. (1996) 467.
[7] K. Tsukagoshi, Y. Okumura, H. Akasaka, R. Nakajima, T. Hara, Anal. Sci. 12 (1996) 869.
[8] L. Saunders, J. Perrin, D. Gammack, J. Pharm. Pharmacol. 14 (1962) 567.
[9] C. Huang, Biochemistry 8 (1969) 344.
[10] N. Oku, D.A. Kendall, R.C. MacDonald, Biochim. Biophys. Acta 691 (1982) 332.
[11] A.D. Bangham, M.M. Standish, J.C. Watkins, J. Mol. Biol. 13 (1965) 238.
[12] Y. Murakami and J. Sunamoto, Koso - Seitaimaku Modelu no Kagaku (Chemistry of Enzyme - Biomembrane Model), Nanko-do, Tokyo, 1981, Ch. 3, p. 105.
[13] K. Kubotsu, S. Matsuura, I. Sakurabayashi, Clin. Chem. 38 (1992) 808.
[14] Y. Ishimori, K. Rokugawa, Anal. Chim. Acta 284 (1993) 227.
[15] F. Szoka, D. Papahadjopoulous, Proc. Natl. Acad. Sci. USA 75 (1978) 4194.
[16] H. Schott, D.V. Cunow, H. Langhals, Biochim. Biophys. Acta 1110 (1992) 151.