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Biochimica et Biophysica Acta 1280 (1996) 149–154

BBA Biochimica et Biophysica Acta

Effect of serum protein binding on real-time trafficking of liposomes with different charges analyzed by positron emission tomography

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Received 21 August 1995; revised 13 November 1995; accepted 23 November 1995

Abstract

Liposomes have been used as carriers of various materials and as tools for gene transfer: for the latter purpose, positively charged liposomes are usually used. To evaluate the stability in the presence of serum and the in vivo behavior of such liposomes as well as those aspects of neutral and negatively charged liposomes, we investigated liposomal agglutinability in the presence of serum, serum protein binding to these liposomes, and real-time liposomal trafficking by a non-invasive method using positron emission tomography (PET). Liposomes composed of dipalmitoylphosphatidylcholine, cholesterol without or with charged lipid were prepared in the presence of mannitol, and the turbidity change in the presence of serum was determined. Turbidity increase was not observed for so-called long-circulating liposomes, i.e., liposomes modified with glucuronic acid or with poly(ethylene glycol), or for negatively charged liposomes containing dicetyl phosphate (DCP), phosphatidylglycerol, or phosphatidylserine. On the contrary, a significant turbidity increase was observed when positively charged liposomes modified with stearylamine, stearyltrimethylammmonium chloride or 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl bromide (DMRIE), which is known as a component of liposomes for gene transfer, were used. These liposomes were found to have bound a high amount of serum proteins after separation of unbound serum proteins by use of a spin column. The liposomal trafficking in vivo was determined for three kinds of liposomes, i.e., liposomes with DMRIE, those with DCP, and those without charged lipids. These liposomes were prepared in the presence of 2-[^{18}F]fluoro-2-deoxy-D-glucose ([2-^{18}F]FDG), and the [2-^{18}F]FDG-labeled liposomes were administered to mice to perform PET scans. Positively charged liposomes containing DMRIE showed high accumulation in the liver compared with neutral and negatively charged liposomes. Since DMRIE-liposomes tended to aggregate in the presence of serum, and to be associated with serum protein, these characters may lead to the high uptake of DMRIE-liposomes by the liver.

Keywords: Liposome; Opsonization; Positron emission tomography (PET); Drug delivery system; Gene transfer

1. Introduction

Liposomes can encapsulate various materials and have been used as drug carriers. Many previous studies have demonstrated the enhanced efficacy of encapsulated drugs and the reduction of the side effects of drugs so entrapped [1,2]. The clearance of liposomes from the circulation is known to occur mainly by the trapping of liposomes in the reticuloendothelial system, such as liver and spleen, and to be greatly affected by liposomal size, charge, and lipid composition. In the field of drug delivery systems, neutral or negatively charged liposomes are commonly used.

On the other hand, liposomes are also being explored as suitable carriers of gene in the field of gene therapy. Some cationic liposomes have been used as a tool of gene transfer [3,4]. For transferring genes in vivo, DNA-liposome complexes have been revealed to be as useful as replication-defective retroviral vectors, though the precise biodistribution of these liposomes has not been examined [5]. Furthermore, gene transfer via liposomes can avoid the

Abbreviations: DCP, dicetyl phosphate; DMRIE, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl bromide; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; FBS, fetal bovine serum; [2-^{18}F]FDG, 2-[^{18}F]fluoro-2-deoxy-D-glucose; PBS, phosphate-buffered saline; PEG-PE, poly(ethylene glycol)phosphatidylethanolamine; PET, positron emission tomography; PGlc, palmityl-D-glucoside; PGlcUA, palmityl-D-glucuronide; PS, phosphatidylserine; SA, stearylamine; STAC, stearyltrimethylammmonium chloride.

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SSDI 0005-2736(95)00283-9

incorporation of DNA into the host genome; this character is useful for the purpose of transient expression of gene products, such as for suppression of inflammatory diseases. Liposomal DNA can be administered via vein, artery, peritoneal cavity, airway, and so on. The intravenous route is attractive if the target can be controlled. The trafficking of such positively charged liposomes in vivo has, however, not been examined precisely.

In the present study we investigated the stability in serum of liposomes containing various modifiers including those for gene transfer, by means of liposomal agglutinability and serum protein binding to these liposomes. Furthermore, the real-time trafficking of three kinds of liposomes with different charges was determined by use of positron emission tomography (PET); the method was recently established by us to determine the liposomal trafficking in vivo non-invasively [6]. The present study indicates that positively charged liposomes are easily trapped by liver, since they tend to aggregate in the presence of serum and to bind serum proteins that may opsonize the liposomes. The data suggest that some modification of liposomes might be necessary for systemic injection of cationic liposomes when the target is not the liver, although differential trafficking may occur when DNA is bound to these liposomes.

2. Materials and methods

2.1. Materials

1,2-Dimyristyloxypropyl-3-dimethylhydroxyethyl bromide (DMRIE) was synthesized according to the method described earlier [7]. Palmityl-D-glucuronide (PGlcUA) was synthesized as previously described [8], and palmityl-D-glucoside (PGlc) was synthesized according to a similar procedures. Poly(ethylene glycol)-conjugated distearoylphosphatidylethanolamine was prepared as described before [9], with PEG having an average molecular weight of 2000. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were the products of Nippon Fine Chemical Co. (Hyogo, Japan). Dicetyl phosphate (DCP), stearylamine (SA), and cholesterol were purchased from Sigma (St. Louis, MO). Stearyltrimethylammonium chloride (STAC) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). BioGel A-15m (200–400 mesh) was obtained from Bio-Rad Laboratories, Inc. (Tokyo, Japan); and fetal bovine serum (FBS), from JRH Biosciences (Lenexa, KS).

2.2. Preparation of liposomes

Liposomes were prepared as follows: DPPC and cholesterol (10:10 in a molar ratio), or DPPC, cholesterol, and modifying lipids (uncharged PGlc or various charged lipids) in a molar ratio of 10:10:1 were dissolved in chloroform/methanol, dried under reduced pressure, and stored in vacuo for at least 1 h. After hydration of the thin lipid film with 0.3 M mannitol (containing 10 mM DPPC), the resulting liposomal solution was frozen in liquid nitrogen and thawed for three cycles. Then the liposomes were extruded through a polycarbonate membrane filter having 400-nm, 200-nm, and 100-nm pores (Nuclepore) three times for each pore size.

Liposomes for the PET analysis were prepared as described previously [6]. In brief, DPPC and cholesterol with DMRIE or DCP (10:10:1 as a molar ratio), or without charged lipid were dissolved in chloroform and dried under reduced pressure. After hydration of the thin lipid film with 1.0 ml of 0.45 M sodium gluconate, the resulting liposomal solution was mixed with 2.0 ml of [2-^{18}F]FDG solution; and three cycles of freeze-thawing using liquid nitrogen was performed for trapping of the radioactive chemical by the liposomes. Then the liposomes were extruded three times through a polycarbonate membrane filer having a 100-nm pore size. To remove the untrapped [2-^{18}F]FDG, We diluted the liposomes with saline and centrifuged them at 180000 × g for 20 min.

2.3. Assay of liposomal aggregation in the presence of serum

Liposomes prepared in 0.3 M mannitol were incubated in PBS or in 50% FBS for 30 min at 37°C (final concentration of liposomes was 0.5 mM as DPPC). Liposomes were also incubated for a week at 4°C to determine their stability during storage. The turbidity of the liposomal solution was determined at 450 nm, and relative turbidity compared with that in mannitol was calculated.

2.4. Determination of serum protein binding to liposomes

100 μl of liposomes prepared in 0.3 M mannitol was incubated with 100 μl of FBS at 37°C for 30 min. Then a 100-μl aliquot of the reaction mixture was applied to the spin column, which was prepared according to a published method [10] that was slightly modified as follows: 1-ml tuberculin syringes plugged with 30 mg of glass wool were filled with BioGel A-15m equilibrated with PBS and packed by centrifugation at 800 rpm for 30 s. The column was eluted with every 100-μl volumes of PBS by a centrifugation at 500 rpm for 30 s. Complete separation of liposomes from free serum proteins was confirmed by separate elution of liposomes and FBS. The liposomal fraction, i.e., the void volume, was collected; and the amounts of protein and phospholipids were determined with a protein assay kit (Bio-Rad) and by phospholipid phosphorus assay, respectively.

2.5. PET analysis of liposomal trafficking

Seven-week-old Balb/c male mice (Japan SLC Inc.) weighing 25–30 g were anesthetized with sodium pento-

barbital and injected into a tail vein with positron-labeled liposomes. The amount administered was 9 μmol as total lipids, and about 1.85 MBq. The emission scan was started immediately after injection and performed for 120 min with an animal PET camera (Hamamatsu Photonics, HSR-2000) having an effective slice aperture of 3.25 mm and resolution of 2.7 mm. Transmission scans were obtained by use of an 18.5 MBq ^{68}Ge/Ga ring source for attenuation correction before the liposomal injection. The radioactivity in the form of coincident gamma photons was measured and converted to Bq/cm^3 of tissue volume by calibration after correction for decay and attenuation. A time-activity curve was obtained from the mean pixel radioactivity in the region of interest (ROI) of the PET images.

3. Results

3.1. Aggregation of and serum protein binding to liposomes with different charges

Hepatic and splenic uptake of liposomes depends on liposomal size. In fact, larger sized liposomes are more easily taken up by liver than the smaller ones. Although the liposomes used here were sized by extrusion method, if the aggregation of liposomes would occur in the presence of serum, it might cause liver trapping of the liposomes. Therefore, we determined the agglutinability of the liposomes containing variously charged lipids. As shown in Fig. 1a, neutral liposomes tended to make aggregates in PBS, whereas the other liposomes did not do so in the PBS. After a week of incubation at 4°C, neutral liposomes formed a visible precipitate which could be easily resuspended by mechanical shaking.

The agglutinability of positively charged liposomes markedly increased in the presence of serum, as shown in Fig. 1b. Neutral and negatively charged liposomes also showed enhanced agglutinability in the presence of serum. In contrast, glucuronic acid- or PEG-modified liposomes did not aggregate in the presence of serum.

The binding of serum proteins, so-called opsonins, to the liposomal surface is another important factor for liposomal uptake by the liver and spleen. Liposomes having a long-circulating life are known to associate with a small amount of serum proteins, whereas liposomes associated with a large amount of serum proteins are easily trapped by the liver and spleen [11]. Thus, we determined the serum protein binding to the liposomes by the spin column procedure for separating protein-bound liposomes from free serum proteins. As shown in Fig. 2, serum protein binding was marked when positively charged liposomes were incubated in FBS. This characteristic may be responsible for the high agglutination of positively charged liposomes in the presence of serum.

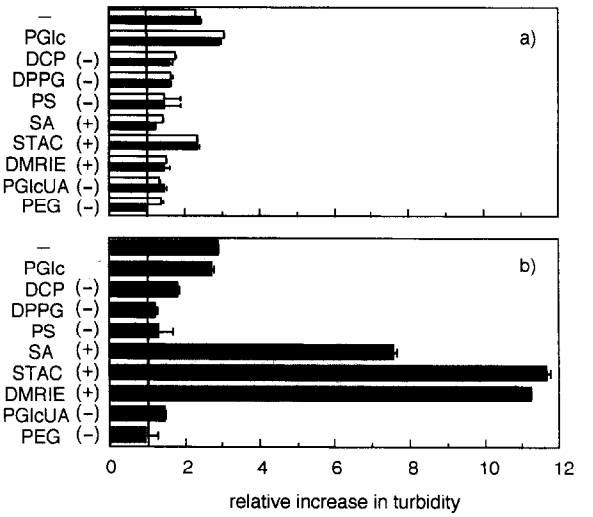

Fig. 1. Liposomal agglutinability in the presence and absence of serum. (a) Liposomes composed of DPPC, cholesterol, and modifying lipids (10:10:1 as a molar ratio) as indicated in the figure were incubated at 4°C for 1 week (open bars) or at 37°C for 30 min (closed bars) in PBS. The relative turbidity at 450 nm against that in 0.3 M mannitol solution is shown. (b) Similar procedures as used in 'a' were performed except that the liposomes were incubated at 37°C for 30 min in the presence of 50% FBS. The net charge of liposomes is shown in parentheses.

3.2. Trafficking of liposomes with different charges determined by PET

Next we determined the trafficking of three kinds of differently charged liposomes. Liposomes containing DMRIE (positively charged liposomes) or DCP (negatively charged liposomes) as well as those without charged lipids

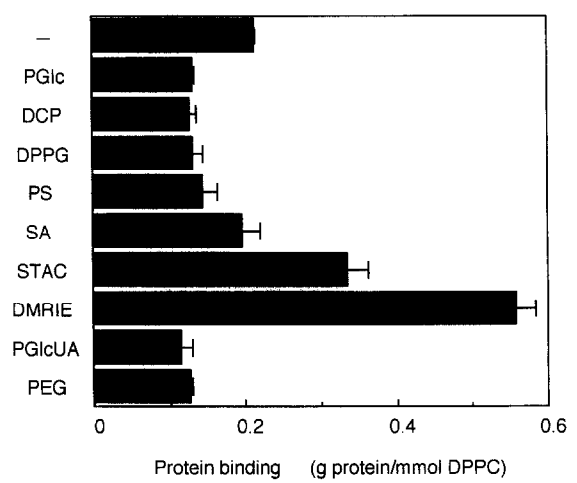

Fig. 2. Serum protein binding to liposomes with various compositions. Liposomes composed of DPPC, cholesterol, and modifying lipids (10:10:1 as a molar ratio) as indicated in the figure were incubated at 37°C for 30 min in the presence of 50% FBS. Then the spin column procedure was performed for separating the liposomal fraction from unbound serum proteins as described in Section 2. The amount of protein and DPPC content in the liposomal fraction were determined and expressed as g protein bound per mmol DPPC.

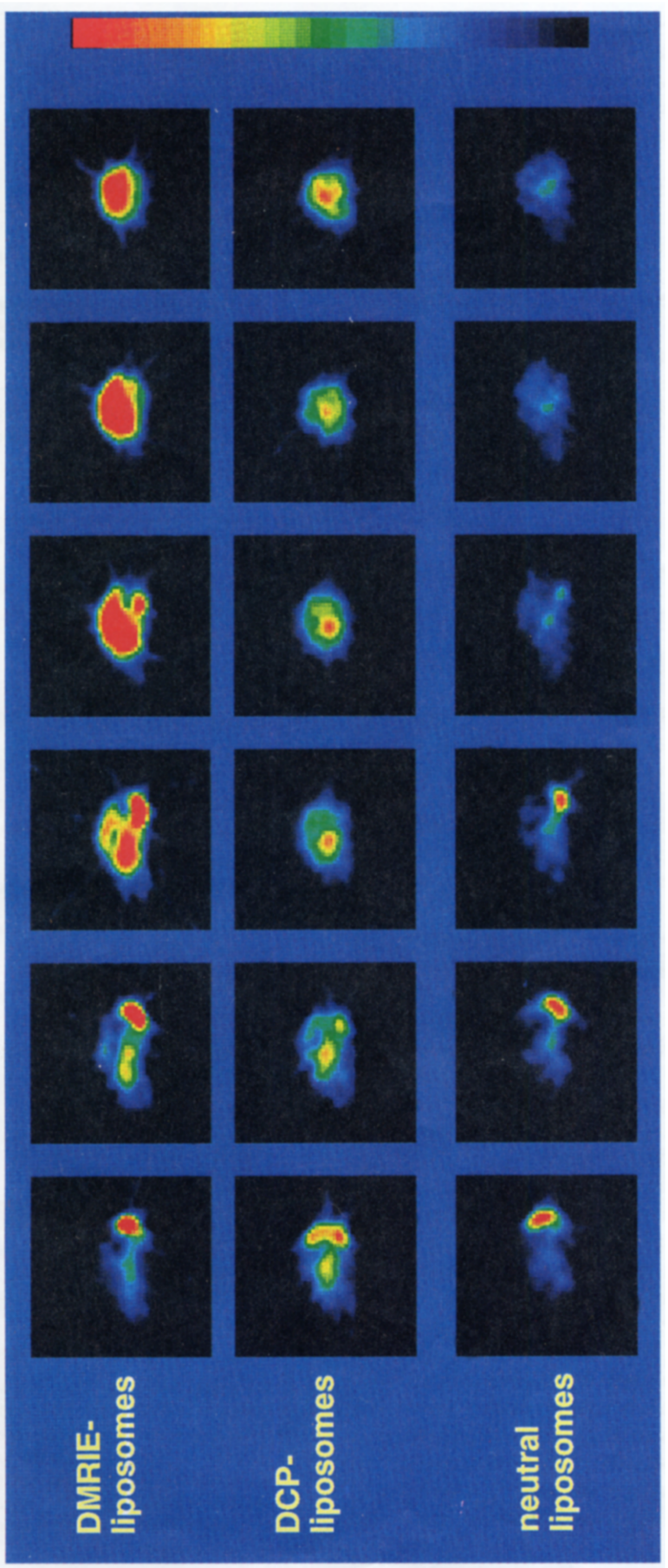

Fig. 3. Biodistribution of [2-^{18}F]FDG-labeled liposomes with various charges imaged by PET. Liposomes composed of DPPC and cholesterol with DMRIE (upper panel), with DCP (middle panel) or without charged lipid (lower panel) were prepared at a molar ratio of 10:10:1 in the presence of [2-^{18}F]FDG and extruded through 100-nm pores as described in Section 2. The [2-^{18}F]FDG-labeled liposomes were injected intravenously into seven-week-old BALB/c male mice, and emission scan was performed. PET images on slices 6 to 11, from tail side (left) to head side (right), are shown where the slice aperture was 3.25 mm. PET images show the accumulation of liposomes during the period of 30 to 60 min after injection.

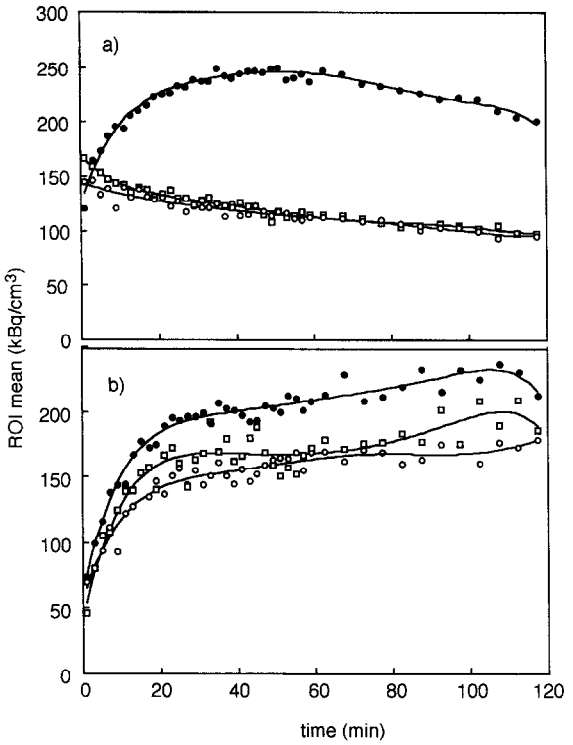

Fig. 4. PET determination of hepatic and splenic accumulation of liposomes with various charges. Liposomal trafficking was determined by PET as described in Section 2 and in the legend of Fig. 3. Time-activity curves of ^{18}F in liver (a) and spleen (b) were obtained after injection of liposomes modified with DMRIE (●), with DCP (○) or without charged lipid (□).

were positron labeled with [2-^{18}F]FDG. Then the PET analysis of liposomal trafficking was performed. Fig. 3 shows the biodistribution of these liposomes in mice during the period of 30 to 60 min after injection. The PET images show the coronal planes of liver and spleen. Slices 8–11 correspond to the liver location; and the right side of slices 6 and 7, to the spleen. Intense accumulation of DMRIE-liposomes shown in the 8–10th slices indicates the liver accumulation of these liposomes. On the contrary, neutral and negatively charged liposomes did not accumulate in the liver compared with the positively charged ones. Accumulations of liposomes in lungs, kidneys and other residual tissues were not observed by PET imaging. Liposomal FDG was shown not to leak out during a 2-h incubation in fetal bovine serum [6], suggesting that the images obtained here indicate the trafficking of liposomes and not any free [2-^{18}F]FDG released from the liposomes.

Fig. 4 shows the time-activity curves of liposomal accumulation in the liver and spleen. The liver accumulation of DMRIE-liposomes was different from that of neutral and DCP-liposomes: DMRIE-liposomes accumulated to a high level time-dependently in the liver, up to 40 min, whereas the other liposomes showed no such increase in the liver accumulation. Liposomal accumulation in spleen was similar among the three types of liposomes, although the highest accumulation was observed when DMRIE-liposomes were injected.

4. Discussion

Liposomes have been used as drug carriers in the field of drug delivery system technology, since they consist of non-toxic biomolecules and they can encapsulate both hydrophilic and hydrophobic materials. Clearance and biodistribution of liposomes of various size, charge, and composition have been determined; however, real-time examination by a non-invasive method has not been done much. We recently developed such a method by use of positron emission tomography [6]. This method might be useful for evaluating liposomes as drug carriers. Therefore, we determined the trafficking of liposomes with various compositions in relationship to the their agglutinability in the presence of serum and to the serum protein binding to them.

With respect to liposomal agglutinability in PBS, neutral liposomes tended to make aggregates, suggesting that charge repulsion prevents liposomal aggregation. Interestingly, more marked aggregation of positively charged liposomes was observed in the presence of FBS. This evidence may relate to the binding of serum proteins to the liposomal surface, as was shown in Fig. 2.

The PET study showed that positively charged liposomes tend to accumulate in the liver and spleen to a much greater extent compared with neutral or anionic liposomes. Although the reason for this high accumulation in the reticuloendothelial system (RES) of the positively charged liposomes is not fully understood, both aggregation of liposomes in the presence of serum and opsonization of them by serum proteins may cause RES trapping of these liposomes. In fact, RES-avoiding liposomes were reported to be associated with fewer serum proteins than liposomes with short half-lives in the bloodstream [11]. Our present result also showed that two types of long-circulating liposomes, i.e., PEG- and glucuronide-modified liposomes, neither aggregated in the presence of serum nor bound a high amount of serum proteins. As positively charged liposomes were reported to activate the complement system via the alternative pathway [12], such liposomes might not be suitable as a drug carrier when the target is not the RES.

The PET analysis presented here also indicated that the hepatic trapping of liposomes reached plateau at about 40 min after injection, while splenic trapping increased time-dependently more slowly to its maximum. This tendency well correlates with reported evidence that liposomal trapping in RES was saturable at first in the liver and then in the spleen [13].

By the way, recent progress in the field of gene transfer indicates that cationic liposomes are useful tools for delivering genes into cells. 2,3-Dioleyloxypropyl-1-trimethyl-

ammonium bromide (DOTMA), being the trademark Lipofectin, has already been commercialized [14]. After development of Lipofectin, 3β-(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol (DC-chol) [15] and DMRIE [7] were developed for enhancing transfer efficiency of genes. In this study we used DMRIE as one of the modifiers of liposomes, since the biodistribution data on these liposomes may be helpful for actual use of these liposomes in the field of gene transfer.

Although the positively charged liposomes are not favorable as a drug carrier, as shown in the present study, cationic liposomes might have potential as a tool for gene transfer. Since the actual trafficking of cationic liposome-DNA complexes might be different from that of positively charged liposomes without DNA, the DNA could reduce the positive charge. Furthermore, although the complex has large size, it is possible that the size is smaller than the aggregation form of the positively charged liposomes without DNA in the serum. We are now planning to examine these possibilities. In the present study, we used DPPC as a main component of liposomes. Dioleoylphosphatidylethanolamine, however, is usually used as a main component of liposomes for gene transfer. This difference may also be possible to affect the liposomal trafficking. We used only DMRIE-containing liposomes in this experiment. To evaluate the trafficking of DOTMA- and DC-chol-containing liposomes may also be interesting.

Acknowledgements

We thank Mr. Shingo Nishiyama and Mr. Takeharu Kakiuchi for their technical assistance. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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