Liposomes Prepared from Synthetic Amphiphiles. I. Their Technetium Labeling and Stability

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In order to apply liposomes prepared from synthetic amphiphiles containing amino acid residues to radiopharmaceuticals, their labeling with ^{99}Tc or ^{99m}Tc and stability in saline or in serum were investigated. These liposomes were highly labeled by embedding stearylamine-diethylenetriamine pentaacetic acid as a ligand of technetium. The labeling was more efficient at pH 7.0 than at pH 4.0 or 8.5. Among these technetium-labeled liposomes tested, liposomes containing the alanine residue were stable in saline or in 50% serum at 37°C for at least 24 h, in contrast to liposomes (phosphatidylcholine : cholesterol = 1 : 1 molar ratio) whose stability had been enhanced by adding cholesterol.

Keywords liposome; amphiphile; technetium labeling; stability; radiopharmaceutical

Introduction

Numerous studies have been reported on the use of liposomes as a model of biomembranes, and as carriers of drugs and radiopharmaceuticals. However, if liposomes are to be utilized as a carrier for delivering therapeutic or diagnostic agents, major problems still remain to be solved, that is, the stability and targeting of the liposomes *in vivo*. Kirby *et al.*[1] suggested that the control of liposomal stability by adjusting the cholesterol content in liposomes may help in the design of liposomes for effective use in biological systems *in vivo* and *in vitro*. Takada *et al.*[2] demonstrated the enhancement of stability and the generation of tissue specificity of liposomes by coating them with polysaccharide derivatives. On the other hand, since Kunitake and Okahata[3] first reported in 1977 that amphiphiles form liposomes (vesicles), as well as naturally occurring phospholipids, new amphiphilic compounds have been synthesized and their behavior in membrane models has been investigated.[4]

Among various amphiphiles already reported, we selected the following three amphiphiles as candidates to develop new radiopharmaceuticals; *N,N*-didodecyl-N^{α}-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide ($N^+C_5Ala2C_{12}$),[5] *N,N*-didodecyl-N^{α}-{6-[dimethyl(2-carboxyethyl)ammonio]hexanoyl}-L-alaninamide bromide ($CAC_2N^+C_5Ala2C_{12}$)[5] and *S*-{1-carboxy-2-[(2,3-bishexadecyloxy)propoxy]carbonyl}ethylhomocysteine ($HcyM^-G2C_{16}$).[6]

Liposome-forming phospholipids or amphiphiles are known to have a tripartite structure, that is, a hydrophobic aliphatic double chain, a hydrophilic head group and a so-called hydrogen belt between the hydrophobic and hydrophilic regions. It was reported by Murakami *et al.*[5] that $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ vesicles, which contain an alanine residue in the hydrogen belt, form stable single-walled vesicles upon sonication in an aqueous medium. Newmann and Ringsdorf[6] found that $HcyM^-G2C_{16}$ liposomes were converted to peptide liposomes by the use of water-soluble carbodiimide as a condensing agent, suggesting the formation of more stable liposomes. Peptide liposomes should have the advantage of being biodegradable. Besides the stability mentioned above, we were interested in the difference of surface charge based on the structure of the hydrophilic region of these liposomes, because the surface charge of liposomes is known to influence the tissue distribution *in vivo*.

This paper deals with the labeling of three synthetic liposomes with ^{99}Tc and ^{99m}Tc for the purpose of applying them as radiopharmaceuticals. Liposomes prepared from a synthetic amphiphile are hereinafter referred to as synthetic liposomes, and liposomes from phosphatidylcholine and cholesterol as natural liposomes.

Materials and Methods

Materials Main reagents for the synthesis of amphiphilic compounds were as follows: di-*n*-dodecylamine (Kanto Chemical Co., Tokyo), Boc-L-alanine (Peptide Institute Inc., Osaka), DL-homocysteine (Sigma Chemical Co., St. Louis, Mo.), stearylamine (Tokyo Kasei Kogyo Co., Tokyo), 2,3-dihydropyran (Kanto Chemical Co. Tokyo). Phosphatidyl choline and cholesterol were products of Nippon Fine Chemical Co., Hyogo, and Sigma Chemical Co., St. Louis, Mo., respectively. ^{99}Mo-^{99m}Tc generator and $^{99}NH_4TcO_4$ were purchased from Daiichi Radioisotope Lab., Tokyo, and Amersham Co., Amersham, respectively. Fetal calf serum was obtained from Gibco Co., Grand Island, N. Y. Other chemicals used were of guaranteed grade.

Methods. Synthesis of Amphiphiles *N,N*-Didodecyl-N^{α}-[6-(trimethyl-

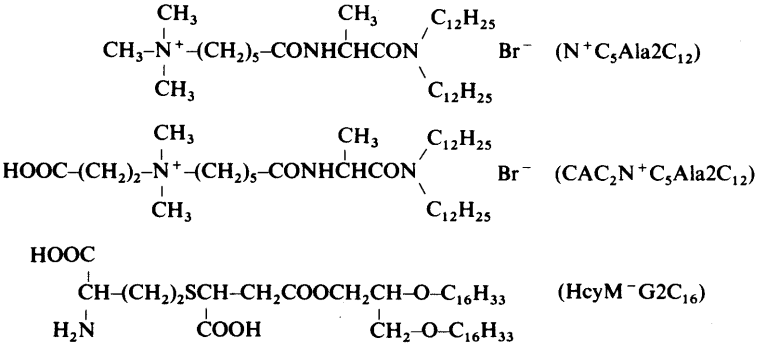

Chart 1. Structure of Amphiphiles

ammonio)hexanoyl]-L-alaninamide bromide ($N^+C_5Ala2C_{12}$) and *N,N*-didodecyl-N^α-{6-[dimethyl(2-carboxyethyl)ammonio]hexanoyl}-L-alaninamide bromide ($CAC_2N^+C_5Ala2C_{12}$) were synthesized by the procedure of Murakami *et al.*,[5] and *S*-{1-carboxy-2-[(2,3-bis-hexadecyloxy propoxy)-carbonyl]ethyl}-homocysteine ($HcyM^-G2C_{16}$) by the procedure of Newmann and Ringsdorf.[6]

Synthesis of Stearylamine-Diethylenetriamine Pentaacetic Acid (SA-DTPA) SA-DTPA, used as a ligand for technetium labeling, was synthesized by the method of Hnatowich *et al.*[7]

Preparation of Synthetic and Natural Liposomes Containing SA-DTPA $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ Liposomes: $N^+C_5Ala2C_{12}$ (5 mM) or $CAC_2N^+C_5Ala2C_{12}$ (5 mM) and SA-DTPA (0.5 mM) were dispersed in 4 ml of physiological saline by a vortex mixer. These dispersions were sonicated for a total of 6 min (1 min sonication with 1 min cooling period, repeated 6 times) with a probe-type sonicator (Tomy, UR200P), giving clear solutions, which were filtered through a 0.2 μm membrane filter (Toyo Roshi, i.d. 25 mm).

$HcyM^-G2C_{16}$ Liposomes: A suspension of $HcyM^-G2C_{16}$ (1.3 mM) and SA-DTPA (0.13 mM) in 4 ml of phosphate-buffered saline (PBS) was sonicated for a total of 15 min (1 min sonication with 1 min cooling period, repeated 15 times) with the sonicator described above, giving a clear solution, which was filtered through a 0.2 μm membrane filter.

Natural Liposomes: A dried thin film of lipid composed of a mixture of phophatidylcholine (6.3 mM), cholesterol (6.3 mM) and SA-DTPA (0.9 mM) was dispersed in 4 ml of saline, sonicated for a total of 10 min (30 s sonication with 1 min cooling period, repeated 20 times) with the sonicator under a stream of O_2-free N_2, giving an almost clear solution, which was filtered through a 0.45 μm membrane filter.

Labeling of Liposomes with ^{99}Tc or ^{99m}Tc ^{99}Tc or ^{99m}Tc-Labeled $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ Liposomes: A mixture of 1 ml of the synthetic liposome solution prepared as described above (2.3.1) and 1 ml of stannous chloride solution (400 μg/ml) was adjusted to pH 7.0 and, after 10 min, 1 mCi of $Na^{99m}TcO_4$ or 0.6 μCi of $NH_4{}^{99}TcO_4$ (2.71 mgTc/ml, 46 μCi/ml) was added. After 40 min, the mixture was filtered through a 0.2 μm membrane filter. The filtrate was chromatographed on a Sephadex G-75 column (1.0 × 30 cm) with saline. The fraction eluted at the void volume was used as ^{99}Tc or ^{99m}Tc-labeled synthetic liposomes.

^{99}Tc or ^{99m}Tc-Labeled $HcyM^-G2C_{16}$ and Natural Liposomes: After mixing 1 ml of $HcyM^-G2C_{16}$ or natural liposome solution with 1 ml of stannous chloride solution (400 μg/ml, pH 7.0), 1 mCi of $Na^{99m}TcO_4$ or 0.3 μCi of $NH_4{}^{99}TcO_4$ (2.71 mgTc/ml, 46 μCi/ml) was immediately added. After 40 min, the mixture was filtered through a membrane filter ($HcyM^-G2C_{16}$ liposomes, 0.2 μm; natural liposomes, 0.45 μm). Each filtrate was chromatographed on a Sephadex G-75 column with PBS ($HcyM^-G2C_{16}$ liposomes) or with saline saturated with N_2 (natural liposomes). The fraction eluted at the void volume was used as ^{99}Tc- or ^{99m}Tc-labeled $HcyM^-G2C_{16}$ or natural liposomes.

Surface Charge of ^{99}Tc-Labeled Synthetic Liposomes ^{99}Tc-Labeled synthetic liposomes were electrophoresed on a cellulose acetate membrane (Sartorius Co. SM11200, 1.5 × 10 cm) saturated with PBS (pH 7.4). The membrane was cut into segments (5 mm wide) and the radioactivity was counted in a liquid scintillation counter (Aloka LSC-661).

Stability of ^{99}Tc-Labeled Liposomes A mixture of 3.5 ml of the ^{99}Tc-labeled liposome solution and 3.5 ml of saline or fetal calf serum was incubated at 37 C. Aliquots of 1 ml taken periodically were chromatographed on a Sephadex G-75 column with saline. The radioactivity of each fraction was measured in a liquid scintillation counter as described above. The stability at each incubation time was determined from the recovery of radioactivity at the void volume in comparison with that at zero time.

Determination of Amphiphiles $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ were determined by the Orange II method,[8] and $HcyM^-G2C_{16}$ by the ninhydrin method. Phosphatidylcholine was assayed by using Phospholipid B-test (Wako Chemicals Co., Osaka).

Results and Discussion

Particle Sizes of Synthetic and Natural Liposomes Particle sizes of liposomes prepared from synthetic amphiphiles or phosphatidylcholine–cholesterol and SA-DTPA were measured under an electron microscope. As shown in Table I, all the particles were similar in size to small unilamellar vesicles (20—50 nm). $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes containing SA-DTPA showed almost the same size as those in the absence of SA-DTPA reported by Murakami *et al.*,[5] indicating that the size of liposomes was little affected by the presence of SA-DTPA.

TABLE I. Size of Liposomes

Liposomes[a]	Mean (nm)[b]
$N^+C_5Ala2C_{12}$ liposomes	75 (25—250)
$CAC_2N^+C_5Ala2C_{12}$ liposomes	50 (25—150)
$HcyM^-G2C_{16}$ liposomes	100 (50—150)
Natural liposomes	100 (50—175)

a) Each kinds of liposomes contains SA-DTPA. *b*) Measured from electron micrographs (Japan Electron Optics Lab. Co., JEM 100S) after negative staining with uranyl acetate ($N^+C_5Ala2C_{12}$, $CAC_2N^+C_5Ala2C_{12}$ and natural liposomes) or sodium phosphotungstate ($HcyM^-G2C_{16}$ liposomes). Numbers in parentheses show the range of size.

Labeling of Liposomes The labeling of synthetic liposomes with radioactive technetium (^{99}Tc or ^{99m}Tc) was investigated by using SA-DTPA embedded in the lipid layer of the liposomes as a ligand for technetium, according to the procedure of Hnatowich *et al.*[7] The labeling yield was determined from the radioactivity eluted at the void volume on a Sephadex G-75 column. The molar ratio of synthetic amphiphile to SA-DTPA was kept constant at 10. The optimal concentration of $SnCl_2$ was 400 μg/ml (data not shown). Since ^{99m}Tc-DTPA, used as a radiopharmaceutical for the kidney, was labeled at pH 4.0—4.5 and Hnatowich *et al.*[7] carried out the ^{99m}Tc-labeling of liposomes (phosphatidylcholine : cholesterol : SA-DTPA = 23 : 3 : 2 molar ratio) at pH 6.6, the labeling of $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes containing SA-DTPA was examined at both pH 4.0 and 7.0. On the other hand, $HcyM^-G2C_{16}$ liposomes were labeled at pH 7.0 and 8.5 because the liposomes aggregated under acidic conditions. In the latter case, the $SnCl_2$ solution was adjusted to pH 7.0 or 8.5 with NaOH solution before addition to the liposome solution.

When the labeled synthetic liposomes were purified by gel-filtration on Sephadex G-75, the elution pattern depended on the eluent used (Fig. 1). This change of the elution pattern could not be explained in terms of the molecular sieve effect of Sephadex gel. One possible reason is that $N^+C_5AlaC_{12}$ liposomes might be partially decomposed by the effect of osmotic pressure in pure water. However, this was not supported by the fact that the liposomes were quite stable in pure water at room temperature.[5] Another possible reason is that there might be some electrostatic interaction or adsorption effect between the liposomes and Sephadex gel in pure water. It is known that Sephadex gel acts as a weak cation exchanger, because the gel has some carboxyl groups.[9] Therefore, it was assumed that $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes having positive charges were adsorbed at the carboxyl groups in the gel through an electrostatic effect. However, when saline was used as an eluting solution, the charge of the carboxyl groups might be counteracted by Na^+ ion, leading to a decrease in the adsorption of $N^+C_5Ala2C_{12}$ liposomes (Fig. 1A). On the other hand, $HcyM^-G2C_{16}$ liposomes were still adsorbed on the gel in saline, but the adsorption was decreased by adding phosphate (Fig. 1B). The effect of

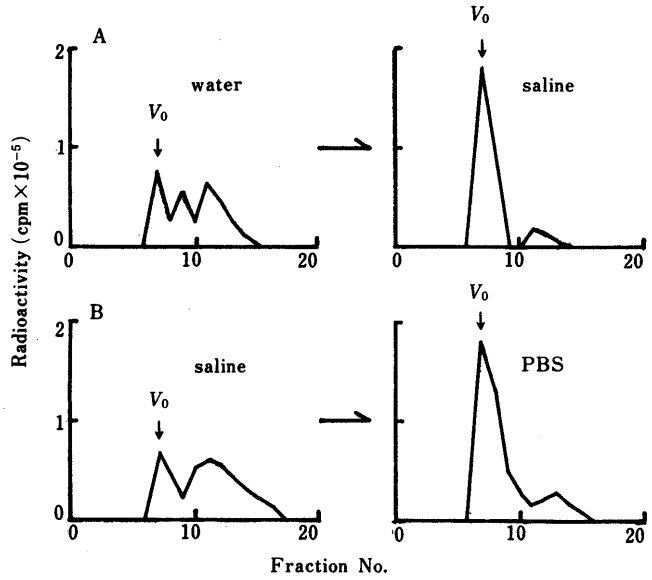

Fig. 1. Effect of Eluent on Elution Profile of ^{99m}Tc-Labeled Synthetic Liposome on Sephadex G-75

Part (A) shows the change of the elution profile of $N^+C_5Ala2C_{12}$ liposomes when the eluent was changed from distilled water to saline. Part (B) shows that of $HcyM^-G2C_{16}$ liposomes when the eluent was changed from saline to PBS. V_0; void volume.

TABLE II. Effect of pH and SA-DTPA on Labeling Efficiency of Liposomes with ^{99m}Tc

Liposomes		Labeling efficiency (%)		
	SA-DTPA	pH 4.0	pH 7.0	pH 8.5
$N^+C_5Ala2C_{12}$ liposomes	+	21.1	42.4	—
	−	13.4	8.3	—
$CAC_2N^+C_5Ala2C_{12}$ liposomes	+	28.2	48.2	—
	−	9.8	10.2	—
$HcyM^-G2C_{16}$ liposomes	+	—	40.4	33.9
	−	—	30.2	28.3
Natural liposomes[a]	+	—	12.1	—

a) Phosphatidylcholine: cholesterol = 1 : 1.

phosphate ion remains unexplained. On the basis of the data shown in Fig. 1, the purification of $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes was carried out by using saline and that of $HcyM^-G2C_{16}$ liposomes by using PBS.

Table II shows that the synthetic liposomes tested were labeled more highly at pH 7.0 than at pH 4.0 or 8.5. The labeling efficiency of $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes was increased significantly when they contained SA-DTPA, whereas that of $HcyM^-G2C_{16}$ liposome showed only a slight increase. The fact that homocysteine itself was highly labeled with technetium[10] seemed to be the reason for the high labeling of SA-DTPA-free $HcyM^-G2C_{16}$ liposomes.

Charge of ^{99}Tc-Labeled Synthetic Liposomes Figure 2 shows the electrophoretic patterns of the three types of ^{99}Tc-labeled synthetic liposomes on cellulose acetate membrane at pH 7.4. Although the mobilities of these liposomes were low, $N^+C_5Ala2C_{12}$ liposomes showed a positive charge, $CAC_2N^+C_5Ala2C_{12}$ liposomes showed a weak positive charge, and $HcyM^-G2C_{16}$ liposomes showed a negative charge. It was expected that these liposomes would

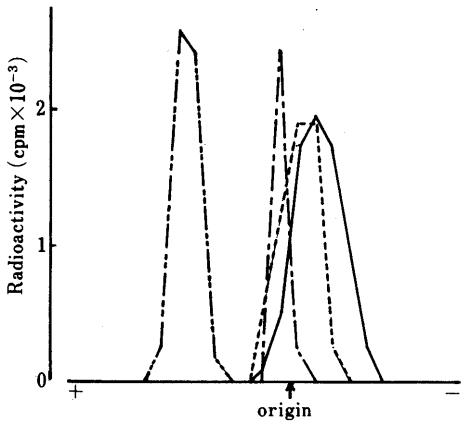

Fig. 2. Electrophoretic Profile of ^{99}Tc-Labeled Liposome on Cellulose Acetate Membrane

^{99}Tc-Labeled liposomes were spotted on a cellulose acetate membrane saturated with PBS (pH 7.4), and electrophoresed at constant current (0.8 mA/cm) for 80 min. ——, $N^+C_5Ala2C_{12}$ liposomes: ------, $CAC_2N^+C_5Ala2C_{12}$ liposomes: —·—, $HcyM^-G2C_{16}$ liposomes: ——·——, $^{99}TcO_4^-$.

circulate *in vivo*, because the pH of blood is 7.35—7.45.

Stability of ^{99}Tc-Labeled Synthetic Liposomes Murakami *et al.*[5] reported that single compartment vesicles ($N^+C_5Ala2C_{12}$ liposomes, $CAC_2N^+C_5Ala2C_{12}$ liposomes, *etc.*, not containing SA-DTPA) were quite stable in aqueous media at room temperature above 25 C owing to the presence of the amino acid residues utilized for the hydrogen belt formation. In order to examine whether ^{99}Tc-labeled synthetic liposomes were affected by ionic strength and by high density lipoprotein (HDL) in serum (reported to cause loss of liposome structure,[11]), the stability of the three types of synthetic liposomes in saline or in 50% serum was investigated in comparison with that of natural liposomes containing SA-DTPA (phosphatidylcholine : cholesterol : SA-DTPA = 7 : 7 : 1 molar ratio). It was reported that intravenously injected cholesterol-rich liposomes (phosphatidylcholine : cholesterol = 1 : 1 molar ratio) remained stable in the blood of animals for at least 400 min.[1]

In the present work, the stability was measured from the recovery of radioactivity eluted at the void volume on Sephadex G-75. This is a measure of the maintenance of ^{99}Tc-labeled liposome structure. However, when the stability is evaluated by this method, the following point must be considered: when ^{99}Tc-labeled liposomes are decomposed in 50% serum, ^{99}Tc-SA-DTPA, $^{99}TcO_4^-$ and $^{99}TcO_2$ may be released as radioactive compounds, and the complex of ^{99}Tc-SA-DTPA or $^{99}TcO_4^-$ with some high molecular components in serum may be eluted at the void volume on Sephadex G-75 gel-filtration. In order to examine this point, SA-DTPA labeled with ^{99}Tc was incubated in 50% serum and the mixture was chromatographed on a Sephadex G-75 column. The radioactivity appeared separately from the void volume fraction and the peak corresponded to that of ^{99}Tc-SA-DTPA. $^{99}TcO_4^-$ was also eluted at nearly the same position. $^{99}TcO_2$, which forms colloidal particles, was not eluted from the gel (data not shown). These results showed that the material eluted at the void volume was ^{99}Tc-labeled liposomes, although the peak of the radioactivity corresponding to ^{99}Tc-SA-DTPA or $^{99}TcO_4^-$ increased gradually with time. Furthermore, the finding that most of the amphiphile used was detected in the

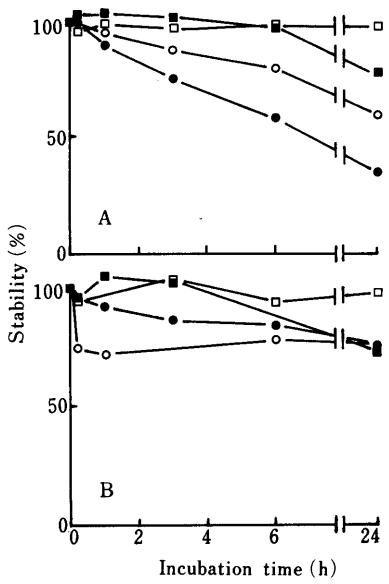

Fig. 3. Stability of ^{99}Tc-Labeled Liposomes in Saline (A) and 50% Serum (B)

^{99}Tc-Labeled liposomes were incubated in saline or 50% fetal calf serum, and gel-filtered on Sephadex G-75. The stability (%) at each incubation time was determined from the recovery of radioactivity at the void volume in comparison with that (100%) at zero time. □, $N^+C_5Ala2C_{12}$ liposomes; ■, $CAC_2N^+C_5Ala2C_{12}$ liposomes; ○, $HcyM^-G2C_{16}$ liposomes; ●, natural liposomes.

void volume fraction (Orange II or ninhydrin reaction procedure) also supported the integrity of the liposomes.

An shown in Fig. 3, the three kinds of ^{99}Tc-labeled synthetic liposomes, especially $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes, were more stable in saline than the natural liposomes, showing that these liposomes were hardly decomposed at physiological ionic strength. In 50% serum, the stability of $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes was almost the same as or greater than that of the natural liposomes. This indicated that the effect of HDL on these synthetic liposomes was low. On the other hand, $HcyM^-G2C_{16}$ liposomes showed initial instability, which was considered to result from the release of technetium bound to the homocysteine residues in the liposomes, rather than partial decomposition. In the Sephadex G-75 assay at 10 min incubation, about 3% of the total radioactivity was detected in TcO_4^- and Tc-labeled SA-DTPA fraction, and about 10% in the gel. The latter fraction might correspond to TcO_2 colloid. Activity (about 10%) was also observed in some fractions right after the void volume, suggesting that some liposomes were partially decomposed. It is expected that this partial decomposition of $HcyM^-G2C_{16}$ liposome may be modified by the conversion of amino acid liposome to peptide liposome using water-soluble carbodiimide.[6]

These results showed that, among the three kinds of ^{99}Tc-labeled synthetic liposomes, $N^+C_5Ala2C_{12}$ and $CAC_2N^+C_5Ala2C_{12}$ liposomes were quite stable in saline and 50% serum at 37 °C for at least 24 h in contrast to natural liposomes whose stability had been enhanced by the addition of cholesterol. The distribution of these ^{99m}Tc-labeled synthetic liposomes in tumor-bearing mice and the interaction with tumor cells will be described elsewhere.

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References

1) C. Kirby, J. Clarke and G. Gregoriadis, *Biochem. J.*, **186**, 591 (1980).
2) M. Takada, T. Yuzuriha, K. Katayama, K. Iwamoto and J. Sunamoto, *Biochim. Biophys. Acta*, **802**, 237 (1984).
3) T. Kunitake and Y. Okahata, *J. Am. Chem. Soc.*, **99**, 3860 (1977).
4) *a*) J. H. Fendler, *Science*, **223**, 888 (1984); *b*) H. Badrer, K. Dorn, B. Hupfer and H. Ringsdorf, *Adv. Polym. Sci.*, **64**, 1 (1985); *c*) D. F. O'Brien, R. T. Klingbiel, D. P. Specht and P. N. Tyminski, *Ann. N.Y. Acad. Sci.*, **446**, 282 (1985).
5) Y. Murakami, A. Nakano and H. Ikeda, *J. Org. Chem.*, **47**, 2137 (1982).
6) R. Newmann and H. Ringsdorf, *J. Am. Chem. Soc.*, **108**, 487 (1986).
7) D. J. Hnatowich, B. Friedman, B. Clancy and M. Novak, *J. Nucl. Med.*, **22**, 810 (1981).
8) G. V. Scott, *Anal. Chem.*, **40**, 768 (1968).
9) J. C. Janson, *J. Chromatogr.*, **28**, 12 (1967).
10) O. Tamemasa, A. Takeda and R. Goto, *Gann*, **75**, 395 (1984).
11) *a*) L. Krupp, A. V. Chobanian and P. I. Brecher, *Biochem. Biophys. Res. Commun.*, **72**, 1251 (1976); *b*) G. Scherphof, F. Roerdrink, M. Waite and J. Parks, *Biochim. Biophys. Acta*, **542**, 296 (1978).