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AN IMPROVED METHOD FOR COVALENT ATTACHMENT OF ANTIBODY TO LIPOSOMES

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A rapid and simple method is described for the incorporation of monoclonal antibody coupled with palmitic acid into liposomes prepared by the reverse-phase evaporation method (Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198). Palmitoyl antibody in 0.15% deoxycholate is added to a liposome suspension after the majority of the organic solvent has been removed by evaporation. Efficient incorporation (over 80%) of palmitoyl antibody occurred without leakage of the encapsulated drug. Native, unmodified antibody did not incorporate under identical conditions. About 50% of the incorporated antibodies could be readily digested by protease, while most of an internal protein marker was not, suggesting that about half of the antibodies were exposed on the outer surfaces of liposomes. Target-specific binding of antibody-liposomes has also been demonstrated in vitro with the RDM-4 lymphoma cells. This method offers a rapid and highly efficient attachment of functional antibody molecules to liposomes with high capture efficiency of drugs, and therefore should be useful in target-specific delivery of drugs mediated by liposomes.

Introduction

Antibody-mediated liposome targeting has become increasingly important in the field of drug delivery. The prerequisite for liposome targeting is to attach antibody molecules covalently to the liposomal membranes without causing leakage or damage of the encapsulated drugs. In order to avoid the exposure of liposomes and their contents to potentially hazardous coupling reagents [1–4], we have developed an alternative procedure for antibody attachment to liposomes [5]. The method involves the derivatization of antibody with plamitic acid and subsequent incorporation of the coupled antibody into liposomes by a detergent-dialysis method. Although a significant number of antibody molecules can be attached to liposomes, the amount of the incorporated antibody exposed at the outer surfaces of liposomes is unknown. Furthermore, the method requires additional steps for the encapsulation of the drug after the formation of liposomes and is thus relatively time-consuming. We report here a rapid method for the incorporation of derivatized antibody into liposomes prepared by the reverse-phase evaporation method [6], which has the added advantage of producing liposomes with high drug capture efficiency.

Materials and Methods

Antibody. Mouse monoclonal antibody to the mouse major histocompatibility antigen, H-2^k, was

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Abbreviations used: NHSP, *N*-hydroxysuccinimide ester of palmitic acid; IgG, immunoglobulin G; PC, phosphatidylcholine; Ara-Cyt, cytosine arabinoside; EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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purified from the culture fluid of a hybridoma line, 11-4.1, as described [5]. Antibody was iodinated with ^{125}I or ^{131}I using chloramine-T to a specific activity of $1 \cdot 10^4$ cpm/μg and $3 \cdot 10^2$ cpm/μg, respectively. α-Bungarotoxin was similarly iodinated with ^{125}I.

Coupling of antibody with palmitic acid. Derivatization of anti-H-2^k with the *N*-hydroxysuccinimide ester of palmitic acid (NHSP) has been described [5]. The molar ratio of NHSP to antibody was 10:1. The coupled antibody was separated from excess NHSP and/or the hydrolysed product, i.e., palmitic acid, by chromatography on Sephadex G-75, equilibrated and eluted with phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/1 mM Na_2HPO_4, pH 7.8) containing 0.15% deoxycholate [5]. The antibody solution was concentrated by ultrafiltration and dialysed against phosphate-buffered saline containing 0.15% deoxycholate. There were about 3–4 palmitoyl chains per IgG molecule on the average (Huang, A., et al., unpublished observation). The final protein concentration was 2–2.5 mg/ml.

Lipids. Egg phosphatidylcholine (PC) was obtained as described [7]. [^{3}H]PC or hexadecyl [^{3}H]cholesterol ether were obtained as described [5] and used to label the liposomal lipids to a final activity of $1 \cdot 10^7$ cpm/mg total lipids. Lipid phosphorus was determined by the method of Ames and Dubin [8]. Cholesterol was purchased from Sigma.

Preparation of liposomes. The reverse-phase evaporation method [6] was used with modification to prepare liposomes. PC (7.7 mg) and cholesterol (3.9 mg) (molar ratio 1:1) were dissolved in 3 ml diethyl ether in a 50 ml round-bottom flask. 0.5 ml phosphate-buffered saline containing water-soluble drugs was added. The mixture was sonicated for 10 min at 0°C in a bath-sonicator (Bronson) until a uniform emulsion was obtained. The excess organic solvent was removed by rotary evaporation at room temperature under reduced pressure until the mixture became a viscous gel. To facilitate the liquification of the gel, 1 ml phosphate-buffered saline was added. The mixture was first vortexed and then incubated for 1–2 h at room temperature. The round-bottom flask was without stopper during this period for continuous evaporation of the residual organic solvent. To the liquified suspension, various amounts of palmitoyl antibody in about 0.5 ml phosphate-buffered saline containing 0.15% deoxycholate was added. The mixture was incubated for 2 h at room temperature before dialyzing for 4 h at 4°C against two changes of 2 ml phosphate-buffered saline to remove the detergent, the residual organic solvent and the majority of the untrapped drugs. The unincorporated antibody and residual free drugs were removed by the Ficoll-flotation method described below.

Ficoll-flotation centrifugation. To separate unincorporated antibody and untrapped drugs from liposomes [9], 2 ml liposome suspension was mixed by vortexting with 2 ml Ficoll solution (25% in phosphate-buffered saline) in a 15 ml Corex tube. 4 ml 10% Ficoll in phosphate-buffered saline was then overlaid, followed by 1 ml phosphate-buffered saline. The tube was centrifuged at 3000 rpm for 30 min in a Sorvall HB-4 swing rotor. The majority of liposomes floated to the interphase between phosphate-buffered saline and 10% Ficoll and was collected using a Pasteur pipet. The 10% and the bottom layers were collected separately to determine the drug entrapment efficiency. To remove contaminating Ficoll, 1 ml phosphate-buffered saline was carefully overlaid on the collected liposome suspension and the tube was centrifuged at 3000 rpm for 15 min. Liposomes in the supernatant were collected and the bottom Ficoll layer was discarded.

Protease digestion of liposomes. Liposomes containing palmitoyl ^{131}I-labeled anti-H-2^k were prepared as described above except that ^{125}I-labeled α-bungarotoxin (spec. act. $9.25 \cdot 10^3$ cpm/μg) was included in the aqueous phase as an internal protein marker. 1 mM EGTA was included in the phosphate-buffered saline buffer used in this experiment to reduce any possible contaminating phospholipase activity in the protease. The liposomes were washed three times with the Ficoll-flotation method to ensure minimal contamination of the untrapped ^{125}I-labeled α-bungarotoxin. The liposome suspension was split into two equal parts. In one part, Triton X-100 (final concentration 0.6%) was added, and phosphate-buffered saline was added to the other. Nonspecific bacterial protease (Sigma type XIV, dialysed against phosphate-buffered saline before use) was added to

both parts to give a final concentration of 0.6% (w/v). After various periods of incubation at 37°C aliquots were taken to assess protein digestion by cold trichloroacetic acid precipitation. Bovine serum albumin (100 μg) was added as carrier protein. In the part of liposome suspension in which Triton X-100 was not present during protease digestion, the same amount of Triton X-100 was added before the precipitation step. Precipitation of proteins was done in 5% ice-cold trichloroacetic acid for 30 min. The precipitate was collected by centrifugation and washed once in cold trichloroacetic acid before counting.

Liposome binding to cells. RDM-4 lymphoma (H-2^k type) and P3-X63-Ag8 myeloma (H-2^d type) cells were cultured in suspension in McCoy's medium supplemented with 10% donor bovine serum (Flow Laboratory). Cells were washed three times in phosphate-buffered saline before use. ^{3}H-labeled liposomes with bound palmitoyl ^{125}I-labeled anti-H-2^k at various concentrations were added to $1 \cdot 10^6$ cells in phosphate-buffered saline. The mixture (final volume 1 ml) was incubated at 4°C for 3 h and then washed three times with phosphate-buffered saline by centrifugation. The cell pellet was dissolved in 1 ml phosphate-buffered saline containing 2% sodium dodecyl sulfate at 4°C overnight and then counted for both ^{3}H and ^{125}I in a scintillation counter. ^{3}H-labeled liposomes without bound antibody or ^{3}H-labeled liposomes plus free, unmodified ^{125}I-labeled anti-H-2^k were used as controls. Measurements were done in duplicate.

Electron microscopy. Antibody-bound or free liposomes were examined with the negative-stain electron microscopy as described [10].

Determination of daunomycin concentration. Daunomycin entrapped in liposomes was assayed by adding 3 ml methanol to 1 ml liposome suspension, and measuring the fluorescence of daunomycin with an Aminco-Bowman spectrofluorometer. Excitation and emission wavelengths were 487 and 558 nm, respectively. The amount of daunomycin in the sample was determined using a standard curve obtained with pure daunomycin in the same solvent system. The standard curve was linear up to $1.2 \cdot 10^{-3}$ μM.

Other materials. [^{3}H]Cytosine arabinoside (Ara-Cyt) and [^{3}H]uridine were obtained from New England Nuclear. Daunomycin was obtained from National Cancer Institute. Ficoll was purchased from Pharmacia and deoxycholic acid from Aldrich. Other chemicals were obtained from commercial sources as highest grade times.

Results

A series of experiments was done in which various amounts of palmitoyl ^{125}I-labeled anti-H-2^k were added to a fixed amount of ^{3}H-labeled liposomes before the residual organic solvent was removed. The degree of antibody attachment was assessed by the Ficoll-flotation method. As shown in Fig. 1, high-efficiency attachment was achieved over a wide range of antibody-to-lipid ratio with an average liposome recovery of 60%. Even at the highest antibody concentration tested (input antibody/lipid = 0.5), the antibody/lipid ratio (0.44)

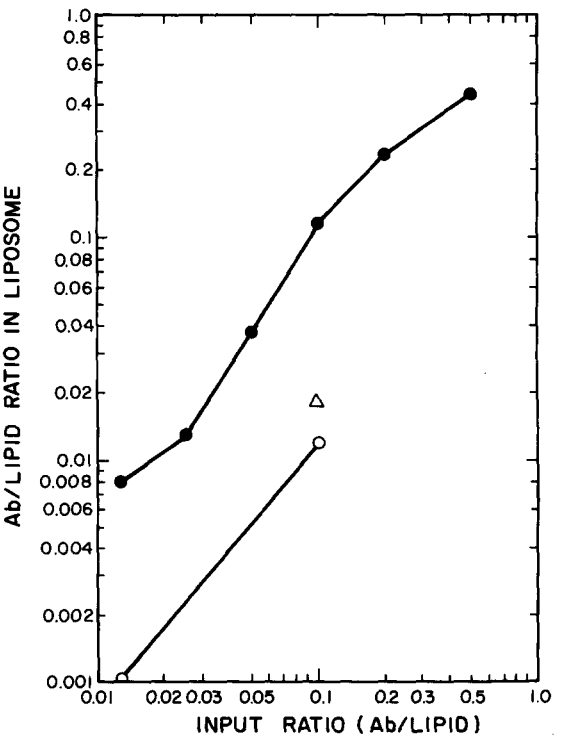

Fig. 1. Attachment of antibody (Ab) to liposomes. Various amounts of palmitoyl antibody (●) or native, unmodified antibody (○) were added to liposomes (total lipid, 1 mg) at indicated weight ratio according to Materials and Methods. Palmitoyl antibody was also added to pre-dialysed liposomes (△).

in the resulting liposomes was still very close to that of the input ratio, indicating no saturation of antibody binding to liposomes at this point. However, the resulting liposomes after flotation on the Ficoll gradient were quite clumpy and difficult to disperse. We therefore chose a lower input ratio of antibody/lipid = 0.1 for routine experiments. No significant antibody attachment was found when the native, unmodified anti-H-2^k was used, indicating that attachment was mediated by the anchorange of the palmitoyl chains into the liposomal bilayers. Interestingly, if liposome preparation was dialysed before the addition of palmitoyl anti-H-2^k, no significant attachment was observed. This observation points out the importance of the residual organic solvent for the antibody incorporation.

We have tested whether the incorporation of palmitoyl anti-H-2^k into liposomes can induce leakage of the entrapped drug. In this series of experiments, radioactive drugs, i.e., [^{3}H]Ara-Cyt and [^{3}H]uridine were included in the phosphate-buffered saline phase of the pre-evaporation mixture, and the lipids were not radioactively labeled. In the case of daunomycin, hexadecyl [^{3}H]cholesterol ether was added to the organic phase, and daunomycin was assayed fluorometrically. As can be seen in Table I, entrapment of drugs range from 13–20% of the total added. Antibody attachment did not significantly change the trapping efficiency, indicating that the insertion of the palmitoyl antibody to liposome bilayers did not cause leakage of the entrapped drug. The entrapped drug and the attached antibody remained liposome-associated for at least 1 week when the liposomes were stored at 4°C in phosphate-buffered saline, as determined by the Ficoll-flotation method.

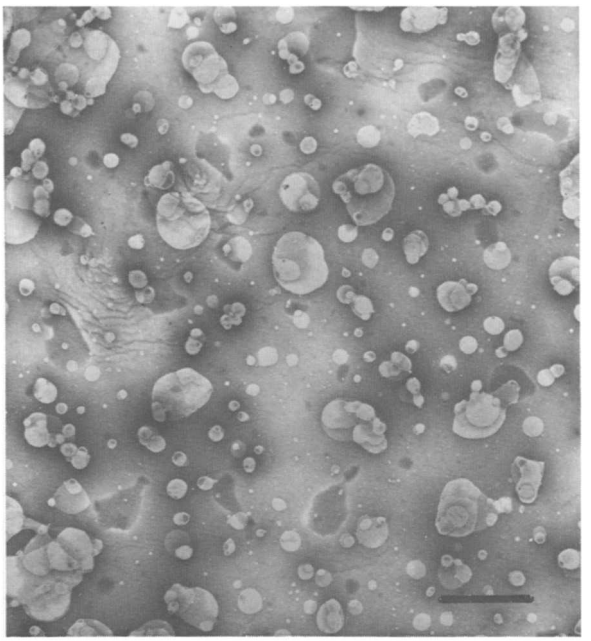

Fig. 2. Negative-stain electron micrograph of antibody attached liposomes. Bar is 1 μm.

Liposomes containing pamitoyl antibody were mixtures of unilamellar (about 60%) and oligola-

TABLE I

DRUG ENTRAPMENT IN LIPOSOMES

Palmitoyl anti-H-2^k was used at protein/lipid ratio = 0.1.

Drug	Palmitoyl anti-H-2^k	Amount of drug added	Amount of drug in liposome	Percentage trapped
[^{3}H]Uridine [a]	–	$1.1 \cdot 10^6$ cpm	$1.7 \cdot 10^5$ cpm	15.5
	+	$1.1 \cdot 10^6$ cpm	$1.4 \cdot 10^5$ cpm	13.0
[^{3}H]Ara-Cyt [b]	–	0.26 mg	0.048 mg	18.0
	+	0.26 mg	0.041 mg	15.8
Daunomycin	–	0.25 mg	0.038 mg	15.2
	+	0.25 mg	0.050 mg	20.0

[a] Tracer amount of [^{3}H]uridine was used. Spec. act. $8.9 \cdot 10^7$ cpm/μg.
[b] Spec. act. $1.2 \cdot 10^6$ cpm/mg.

mellar (about 40%) ones as revealed by negative stain electron microscopy (Fig. 2). The average diameter of liposomes was about 0.15 ± 0.05 μm and 0.45 ± 0.1 μm for unilamellar and oligolamellar liposomes, respectively.

We have used a protease digestion technique to see if the antibody molecules were accessable to protease. A small protein (M_r 8000), α-bungarotoxin, was trapped inside the liposomes to serve as an internal marker for this study. When the liposomes were dissolved by Triton X-100, both palmitoyl anti-H-2^k and α-bungarotoxin were rapidly digested by protease as indicated by the loss of trichloroacetic acid-precipitable radioactivities (Fig. 3). The kinetics of digestion were complex, probably due to heterogeneity of the enzyme activity in the protease used. There was a fast phase of digestion for both palmitoyl anti-H-2^k ($t_{1/2} = 0.15$ h) and α-bungarotoxin ($t_{1/2} = 0.32$), followed by one or two slower phases. Evenually, both proteins were completely digested; less than 10% of the original proteins were left after 20 h. When liposomes were kept intact during digestion about 23% of α-bungarotoxin was rapidly digested with a $t_{1/2} = 0.4$ h and the remaining α-bungarotoxin was protected from protease in the intact liposomes. On the other hand, a larger amount (about 50%) of palmitoyl anti-H-2^k was rapidly digested ($t_{1/2} = 0.15$ h). The remaining antibodies were also digestible at a much slower rate; about 33% remaining at 20 h. These results are consistent with the notion that about 50% of the

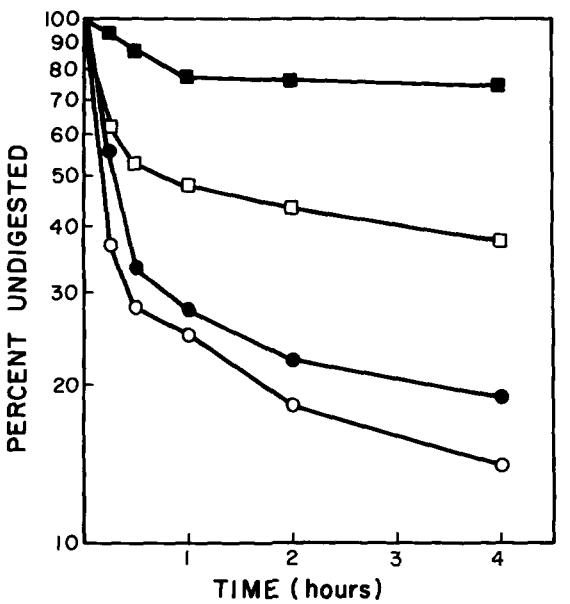

Fig. 3. Protease digestion of antibody attached liposomes. Palmitoyl ^{131}I-labeled anti-H-2^k was attached to liposomes containing ^{125}I-labeled α-bungarotoxin as a trapped marker. Percent of undigested proteins was measured by acid precipitation at various incubation times. ^{125}I-labeled α-bungarotoxin (filled symbols); palmitoyl ^{131}I-labeled anti-H-2^k (open symbols). Digestion was done in the presence (circles) or absence (squares) of Triton X-100.

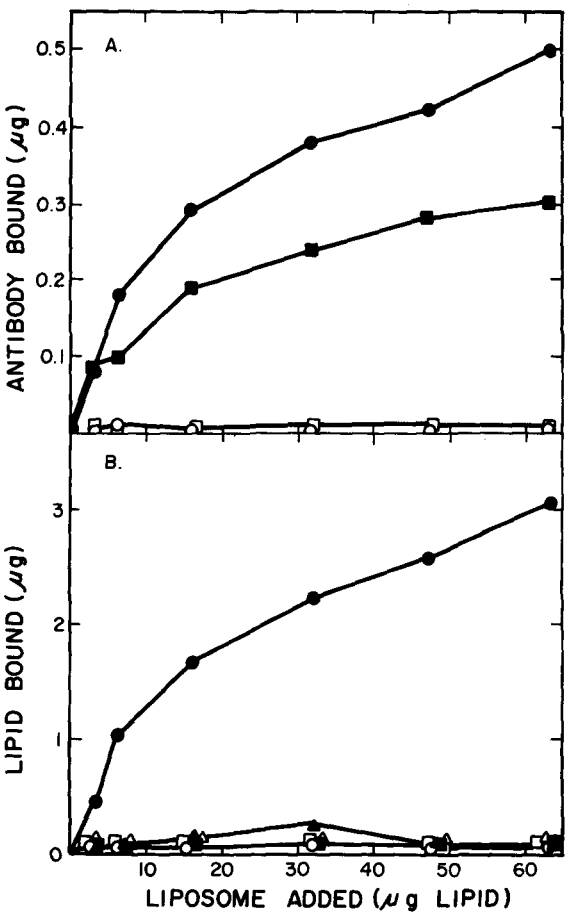

Fig. 4. Binding of liposomes to cells. ^{3}H-labeled liposomes containing palmitoyl ^{125}I-labeled anti-H-2^k (●, ○), protein-free ^{3}H-labeled liposomes (▲, △), and protein-free ^{3}H-labeled liposomes plus unmodified ^{125}I-labeled anti-H-2^k (■, □) were added to $1 \cdot 10^6$ mouse RDM-4 cells (closed symbols) or P3-X63-Ag8 cells (open symbols). The binding of antibody (panel A) and of lipids (panel B) are shown.

liposome-associated antibodies are exposed at the liposome surface and readily accessible to protease. The remaining antibodies are either located at the inner surfaces of the unilamellar liposomes or located at inner liposomes enclosed by larger liposomes in the oligolamellar ones. The small amount of α-bungarotoxin that is rapidly digestible probably represents those nonspecifically adsorbed on the liposome surface.

It is important to show that the liposome-bound antibody is still able to recognise antigen. We have used a mouse lymphoma line, RDM-4, which is derived from a C_3H mouse and contains H-2^k antigens on its surface. The control cell line, P3-X63-Ag8, is a myeloma of the H-2^d type. The binding of palmitoyl anti-H-2^k liposomes to each cell type was measured for both antibody (^{125}I cpm) and liposomal lipids (^3H cpm) (Fig. 4). In controls, protein-free liposomes and liposomes plus native, unmodified antibody were also tested for binding. Protein-free liposomes, with or without free antibody, did not show significant binding to either cell type. Significant liposome binding was observed only when palmitoyl anti-H-2^k was covalently attached. This binding was specific to the RDM-4 cells, since no binding was detected for the P3-X63-Ag8 cells. Furthermore, the binding of the liposome bound anti-H-2^k was about twice as much as that of the free anti-H-2^k. This is probably not due to more antigenic sites exposed on the cell surfaces, and rather reflects the multiple association of antibody molecules with a single binding event, since there were many antibody molecules per liposome. These results clearly indictate that the attached antibody molecules were still capable of binding with antigens and the target-specific binding of liposomes, i.e., liposome targeting, was demonstrated.

Discussion

The present method represents an improvement of our previous precedure in which palmitoyl antibody was incorporated into liposomes by a deoxycholate-dialysis procedure [5]. Using the previous method, one can attach about 40–50 IgG molecules per 1000 Å liposome; however, it is not known what fraction of these molecules are exposed on the outer surfaces of the liposomes. Furthermore, the entrapment of drug can only be carried out by additional steps involving the use of detergent and gel filtration. In the present method, the incorporation of the palmitoyl antibody was done by adding antibody to liposomes with the drugs already encapsulated. Furthermore, a large fraction of the attached antibody molecules are exposed to the outer surfaces of liposomes as indicated by the accessibility of the antibody to protease digestion (Fig. 3). This method is capable of loading liposomes up to an antibody/lipid ratio of about 0.5. Assuming that the number of lipid molecules per 0.15 μm liposome is $2 \cdot 10^5$ [11], this amounts to about 200 exposed antibody molecules per liposome. This level of attachment is equal to or higher than that which can be achieved by most of the other published methods [2,13]. The antibody-bound liposomes prepared with the present method exhibited specific binding properties to target cells (Fig. 4), indicating that the attached antibody molecules were fully functional.

There are other published methods for the covalent coupling of antibody to liposomes. Most of these methods require the exposure of the preformed liposomes to some relatively harsh coupling reagents [1–4], which may result in the leakage and/or alteration of the entrapped drugs. Some of them using glutaraldehyde [1] and carbodiimide [3,12] are likely to produce homo-coupling between liposomes or between antibodies. Some require multiple synthetic and/or purification steps [4,13,14,15]. Others cause significant leakage of the entrapped drug when the modified antibody is incorporated into liposomes [12]. Our approach is fundamentally different in terms of the coupling strategy. Since only antibody is exposed to the coupling reagent, the liposomes and their trapped contents are protected from potential deleterious effects. Furthermore, there is little chance of homo-coupling between liposomes or between antibodies. This procedure can achieve a high level of antibody attachment to liposomes without inducing the leakage of entrapped drugs. Since the reverse phase evaporation method is used in this procedure, drugs can be captured in the liposomes with a relatively high efficiency. The entire procedure starting from the derivatization of antibody with palmitic acid can be completed within 24 h and the resulting liposomes are stable

for at least 1 week. We therefore concluded that liposomes prepared by this method should be well-suited for site-specific drug targeting mediated by antibody.

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