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Convenient Preparation of Tumor-specific Immunoliposomes Containing Doxorubicin

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Two innovative methods to prepare target-sensitive immunoliposomes containing doxorubicin by coupling monoclonal antibodies (mAb DH2, SH1) specific to cancer cell surface antigens (G_{M3}, Le^X) have been developed and are described here. Firstly, liposomes containing N-glutaryl phosphatidylethanolamine (NGPE) were prepared, followed by the encapsulation of doxorubicin by proton gradient. After the encapsulation of doxorubicin, DH2 or SH1 antibodies were conjugated to NGPE in the liposomes (direct coupling). Secondly, liposomes were prepared with NGPE/mAb conjugates by the detergent dialysis method (conjugate insertion), and then doxorubicin was encapsulated by proton gradient. The immunoliposomes prepared by both methods were able to specifically bind to the surface of the tumor cells — B16BL6 mouse melanoma cells and HRT-18 human colonic adenocarcinoma cells. The efficiencies of doxorubicin-entrapping into liposomes prepared by direct coupling and conjugate insertion was about 98% and 25%, respectively. These types of liposomal formulation are sensitive to target cells, which can be useful for various clinical applications.

Keywords: Doxorubicin, Immunoliposome, Target-sensitivity

Introduction

Liposome-mediated delivery of antineoplastic drugs has been considered as one of the most promising procedures for cancer treatment because it is able to reduce adverse side-effects of drugs as well as provide target-sensitivity to drugs (Gregoriadis, 1993). Among anti-neoplastic drugs, doxorubicin, an anthracycline aminoglycoside, is a potent and widely applied anti-cancer drug with a broad spectrum of anti-tumor activity (Carter, 1975; Young *et al.*, 1981). It has been also well-investigated that doxorubicin induces cardiotoxicity (Myers *et al.*, 1977; Olson *et al.*, 1990) as well as serious ill-effect on bone marrow, which restricts its routine clinical usage for cancer treatment. A number of studies have shown that encapsulation of doxorubicin into liposomes markedly reduces the cardiotoxicity of the drug (Rahman *et al.*, 1980, 1986; Gabizon *et al.*, 1982). Therefore, various liposomal formulations of doxorubicin have been extensively studied to be clinically applied for cancer treatment, and now several formulations of liposomal doxorubicin are under clinical trials (Delgado *et al.*, 1989; Treat *et al.*, 1990).

However, conventional doxorubicin-containing liposomes lacking target-sensitivity are rapidly taken up by the reticuloendothelial system (RES) in the liver and the spleen before reaching the intended area (Park and Huang, 1993a,b). The reduced cytotoxicity of doxorubicin would be a result of the sustained release of encapsulated drugs. Thus, a great deal of effort has been made to provide target-sensitivity to liposomal drugs (Ahmad *et al.*, 1993; Vingerhoeds *et al.*, 1996). Those approaches to achieve tumor-specific delivery of liposomal drugs is in combining tumor-specific antibodies to liposomes without lessening the drug-encapsulating capability. However, the previous reports suggested complex conjugation procedures, such as avidin/biotin conjugation to antibodies or phospholipids (Ahmad *et al.*, 1993) or N-[4-(p-maleimidophenyl)butyryl] phosphatidylethanolamine (MPB-PE) conjugation to Fab fragment of immunnoglobulin (Vingerhoeds *et al.*, 1996). Both procedures may not be appropriate for clinical applications because of immunogenicity of avidin/biotin conjugation and low binding affinity of the Fab′ fragment.

In this paper we report two innovative methods to prepare doxorubicin-containing immunoliposomes that specifically bind to cancer cells. Monoclonal antibody DH2 (Dohi *et al.*, 1988) and SH1 (Nakasaki *et al.*, 1989) are derived from ganglioside G_{M3} and Lewis X antigen

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(Le^X), respectively. Ganglioside G_{M3} is aberrantly expressed on the cell surface of B16BL6 mouse melanoma (Dohi, 1988), and Le^X is abnormally expressed on the cell surface of HRT-18 and HT-29, both human colonic adenocarcinoma cells (Otaka *et al.*, 1989). We prepared cancer-specific immunoliposomes using DH2 and SH1 antibodies by two different procedures in terms of antibody-coupling and doxorubicin-encapsulation. The immunoliposomes prepared by both methods were characterized in several ways in order to examine their stability and target-sensitivity.

Materials and Methods

Materials Egg phosphatidylcholine (ePC) was purchased from Doosan S.R.L. (Seoul, Korea). Cholesterol (Chol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Sephadex G-200, and doxorubicin were purchased from Sigma Chemical Co. (St. Louis, USA). N-hydroxysulfosuccinimide (S-NHS) was purchased from Pierce (Rockford, USA). N-glutaryl phosphatidylethanolamine (NGPE) was purchased from Avanti Polar Lipids (Birmingham, USA). Protein A-agarose was purchased from Upstate Biotechnology (Lake Placid, USA).

Hybridoma cell culture and monoclonal antibody preparation Hybridomas producing the monoclonal antibodies against G_{M3} and Lewis X (DH2, SH1, both of mouse IgG_3 type) were generously donated by Dr. Senitiroh Hakomori (North Pacific Research Foundation, Seattle, USA). The hybridoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), streptomycin, and penicillin. The grown hybridomas were inoculated into the peritoneal cavity of mice. The antibodies were purified from mouse ascites fluid by protein A-agarose affinity chromatography.

Tumor cell culture B16BL6 mouse melanoma cells were grown in Minimum Essential Media (MEM) containing FBS 5%, streptomycin, penicillin, and supplemented with 0.1 mM nonessential amino acids, 1 mM vitamin solution, 2 mM L-glutamine, and 1 mM sodium pyruvate. HRT-18, HT-29 human colonic adenocarcinoma cells were grown in DMEM containing 10% FBS, streptomycin, and penicillin. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Preparation of Immunoliposomes Containing Doxorubicin

Method I: direct coupling of antibody DOX was encapsulated by the proton gradient method (Lawrence *et al.*, 1990) and antibodies were directly coupled to the liposomes containing DOX as described by Maruyama (1995) with some minor modifications. EPC/Chol/NGPE (63 : 31 : 6, mole ratio) were hydrated in the presence of 150 mM citrate (pH 4.0) and then extruded through polycarbonate filters (220 nm, pore size). Transmembrane pH gradient was established by titrating the exterior pH to 7.5. Doxorubicin was added to the liposome solution (weight ratio of DOX to lipid, 1 : 5), which was incubated at 60°C for 10 min. After doxorubicin encapsulation, the exterior buffer was changed to a MES buffer (5 mM MES, 0.15 M NaCl, pH 5.5), and 0.05 mol% monoclonal antibodies (DH2, SH1) were added to the liposomes. The antibody/liposome mixture was incubated in the presence of 0.25 M EDC and 0.25 M S-NHS at 4°C for 8–12 h with frequent mixing. Free antibodies and free DOX were separated by Sephadex G-200 chromatography.

Method II: insertion of antibody-conjugate The antibody-NGPE conjugates were synthesized as described by Pinaduwage and Huang (1992). NGPE (0.067 μmole) was dissolved in 100 μl of MES buffer containing 0.01 M octyl glucoside. After the addition of 20 μl of 0.25 M EDC and 0.1 M S-NHS, the resulting mixture was incubated at room temperature for 10 min. The mixture pH was then adjusted to 7.5 and 6.7 nmole of antibodies in 50 mM sodium borate buffer (pH 7.6) was immediately added to the reaction mixture. The reaction mixture was incubated at 4°C for 8–12 h with frequent mixing. The antibody conjugates were mixed with other lipid components mentioned above in the presence of octyl glucoside. The antibody/lipid mixture was dialyzed against 300 mM citrate (pH 4.0) and then extruded through polycarbonate filters (220 nm, pore size). DOX was added into the liposome solution after neutralizing the exterior pH. The reaction mixture was incubated at 37°C for 30 min and then filtrated through Sephadex G-200.

Characterization of immunoliposomes The liposome diameter was measured before and after antibody-coupling or DOX-encapsulation by the laser light scattering using a particle analyzer (Master Sizer MS-20, Malvern, UK). The antibody-coupling to the liposomes was confirmed by SDS-PAGE. Liposomal vesicle formation was verified by negative-stain electron microscopy (Joel 200EX-II, Japan) using 5% uranyl acetate.

Specific binding of the immunoliposomes to B16BL6 mouse melanoma cells, HRT-18, HT-29 human colonic adenocarcinoma cells, and CT26 mouse colorectal carcinoma cells was monitored by immunostaining. The tumor cells were grown on cover glasses in 24-well plates as mentioned above. The tumor cells on the cover glass were washed with PBS several times and then incubated in the presence of 3% paraformaldehyde for 30 min at room temperature. After the fixed tumor cells were completely washed with PBS, an aliquot of the immunoliposomes was then loaded on the cover glass and incubated for 1 h at room temperature. The tumor cells were incubated with FITC/anti-mouse IgG conjugates (Sigma Chemical Co. St. Louis, USA) for 1 h at room temperature and viewed with a fluorescence microscope (Olympus BX50, Japan).

Results

Doxorubicin-containing and antibody-coupled immunoliposomes were successfully prepared by two different methods. After removing the free DOX using column chromatography, the concentrations of lipids and DOX in each fraction were measured by phosphate assay (Marinetti, 1962) and absorbance at 490 nm. Almost all DOX added to the liposomal solution was recovered with

the liposomes prepared by method I (Fig. 1A) In the case of liposomes prepared by method II, about 75% unencapsulated DOX was aggregated and became precipitates during gel-filtration (Fig. 1B). Final DOX encapsulation efficiencies of immunoliposomes prepared by method I and method II were over 98% and about 25%, respectively.

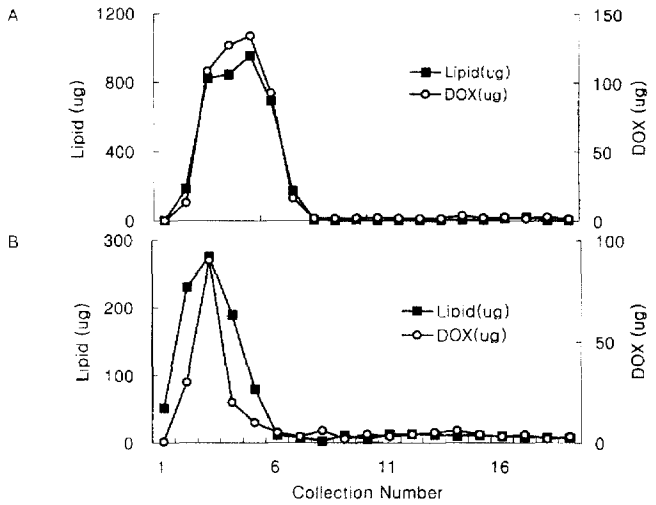

Fig. 1. Efficiency of DOX encapsulated into immunoliposomes prepared by method I (A) and method II (B). Concentrations of lipids and DOX in each faction of gel filtration chromatography were quantified by the phosphate assay and photometric measurement at 490 nm.

In order to view immunoliposomes containing DOX and coupled to antibodies, electron microscope photographs of the liposomes were taken after negative staining with 5% uranyl acetate (Fig. 2). Since the liposomes were sized through polycarbonate filters (220 nm), the size variation was relatively small. Antibody-coupling and DOX-encapsulation did not change the vesicle size of liposomes. The size change of the immunoliposomes was also monitored by light-scattering technique (Table 1). There were only small changes in liposome size after doxorubicin-encapsulation and antibody-coupling (Table 1). The size change of liposomes appeared to be more affected by antibody-coupling than by DOX-encapsulation.

Antibody-coupling to liposomes was verified by SDS-PAGE (Fig. 3). Free antibodies were removed by gel filtration and were quantified by Bradford method (Bradford, 1976). Almost all antibodies added into the reaction solution were coupled to liposomes prepared by both methods: 99% and 95%, respectively (data not shown).

The target-sensitivity of the immunoliposomes prepared by each method was revealed by fluorescence microscopy. The tumor cells (B16BL6 mouse melanoma cells and

Table 1. Size changes of liposomal vesicles during DOX-encapsulation and antibody-coupling.[1]

			(nm ± SD)
Method	After extrusion	After DOX-encapsulation	After IgG-coupling
I	202 ± 45	208 ± 54	250 ± 46
II	198 ± 51	210 ± 49	ND[2]

[1] The liposome diameter was measured by the laser light scattering using a particle analyzer.
[2] Antibody/NGPE conjugates were already incorporated into liposomes before DOX-encapsulation.

HRT18 human colon adenocarcinoma cells) were incubated with the immunoliposomes prepared by method I and then with FITC/anti-mouse IgG conjugates. Free DH2 and SH1 antibodies exhibited strong *in vitro* binding affinity to the surface of B16BL6 and HRT-18, respectively (Figs. 4A,C). DH2- and SH1-immuno-liposomes were also able to bind to the cell surface with somehow reduced binding affinity compared to the free antibodies at the same antibody concentration (Figs. 4B,D). Meanwhile, free DH2 and SH1 antibodies were not able to specifically bind to HRT-18 and B16BL6, respectively (data not shown).

Discussion

It has been well documented that DOX encapsulated in liposomes exhibits reduced cardiotoxicity and enhanced drug efficacy. The improved therapeutic effect is resulted from the sustained release of DOX from RES which rapidly takes up liposomal DOX as soon as it is administered *in vivo*. Hence, the therapeutic effect of DOX administered with liposomes is still limited and its routine subscription should be restricted as in the systemic administration of DOX. Therefore, a number of studies have been focused on more specific targeting of liposomal drugs to target cells because it will be able to significantly reduce the therapeutic dosage for cancer patients, resulting in enhancement of the therapeutic efficacy of DOX . There have been a few studies regarding immunoliposomes sensitive to target tissues or tumor cells (Maruyama *et al.*, 1990, 1995; Pinaduwage and Huang, 1992; Ahmad *et al.*, 1993; Vingerhoeds *et al.*, 1996). However, some reports showed only better targetable immunoliposomes without any drugs and others reported quite complex formulations of immunoliposomes containing DOX to achieve better treatment for cancer. Concerning the immunoliposomes containing DOX, antibody-conjugation procedures in the previous reports were complex, such as avidin/biotin conjugation to antibodies or phospholipids (Ahmad *et al.*, 1993) or MPB-PE conjugation to Fab' fragment of

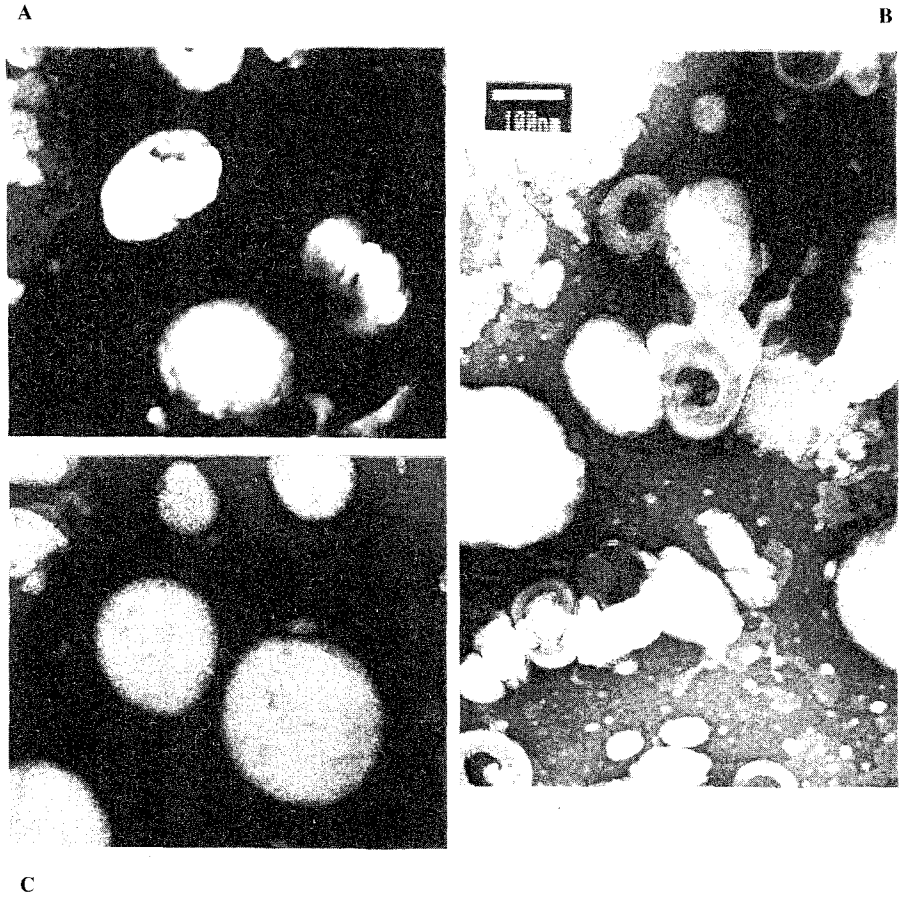

Fig. 2. Electron microscope photographs of ePC/Chol/NGPE (A), ePC/Chol/NGPE/DOX (B), and ePC/Chol/NGPE/DOX/SH1 (C). The photographs were taken after negative staining with 5% uranyl acetate by an electron microscopy.

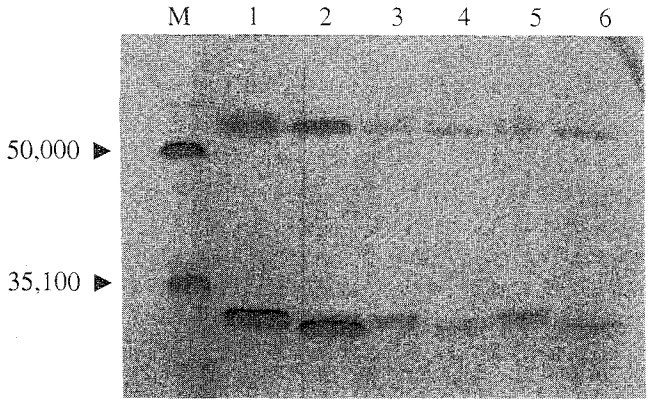

Fig. 3. Antibody-coupling to liposomes. After antibody-coupling and DOX-encapsulation, antibody-coupling was verified by SDS-PAGE. molecular standards (M), free DH2 (1), free SH1 (2), DH2 immunoliposomes (3), and SH1 immunoliposomes (4) prepared by method I, DH2 immunoliposomes (5), and SH1 immunoliposomes (6) prepared by method II.

immunnoglobulin (Vingerhoeds *et al.*, 1996).

In this report we have introduced two convenient methods for DOX-containing liposomes sensitively binding to tumor cells. The liposomes prepared by method I exhibited high efficiency (98%) of DOX-encapsulation (Fig. 1A). Also, we may be able to control antibody density on the surface of liposomes more precisely. An advantage of method II was the convenience in preparation and accurate size control of liposomal vesicles (Table 1). In method II the incubation temperature for DOX-encapsulation had to be decreased to 37°C in order to minimize damage to antibodies coupled to the liposomes. The low DOX-encapsulation of immuno-liposomes prepared by method II may be primarily due to the relatively low incubation temperature. Depending on the experimental conditions and purposes, either method can be chosen to prepare immunoliposomes containing drugs.

The immunoliposomes prepared by both methods were able to sensitively bind to the cell surface of tumor cell, even though they exhibited some reduction of binding affinity compared with the free antibodies (Fig. 4). The decrease in binding affinity of the immunoliposomes may be a result of the fixed orientation of antibodies coupled to the liposomal surface and the steric hinderance induced by the massive size of liposomal vesicles themselves.

Both methods hardly impaired the encapsulation

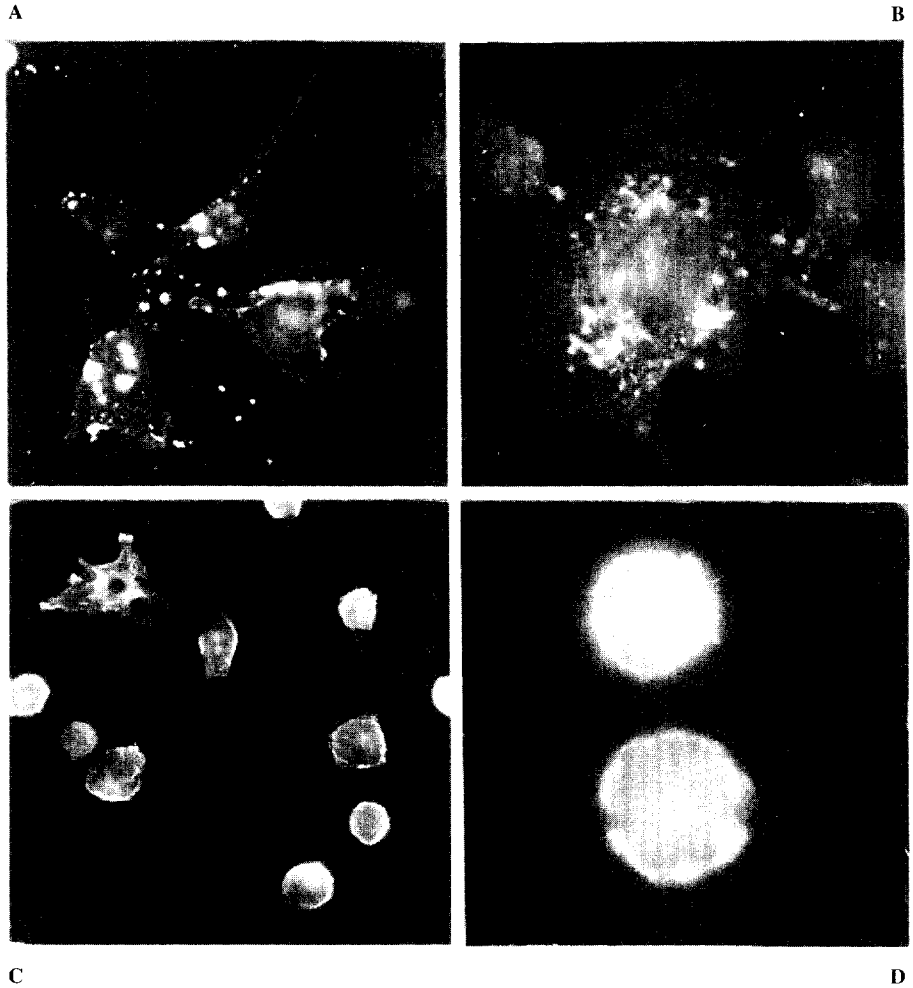

Fig. 4. Tumor-specific binding of immunoliposomes. The fixed B16BL6 mouse melanoma cells were incubated with free DH2 (A) or DH2 immunoliposomes (B), and the fixed HRT-18 tumor cells were incubated with free SH1 (C) or SH1 immunoliposomes (D). The tumor cells were then incubated with FITC/anti-mouse IgG conjugates and viewed by a fluorescence microscope.

efficiency of doxorubicin into the immunoliposomes compared with encapsulation into conventional liposomes. In addition, the binding affinity of antibodies to antigens expressed on the tumor cells was not damaged during the entire processes of liposome preparation. The continued binding affinity of antibody after being coupled to the liposomal surface and the stability of immunoliposomes during the preparation process may imply that both procedures are gentle enough to be broadly utilized for various applications.

In order to provide tumor cell-sensitivity to the liposomes containing DOX, DH2 (Dohi *et al.*, 1988) or SH1 (Otaka *et al.*, 1989) monoclonal antibodies were conjugated to the liposomal surface. It has been suggested that there is no tumor-specific molecules (or structures) of carbohydrates on the tumor cell surface (Nakasaki *et al.*, 1988; Hakomori, 1992). Basically G_{M3} and Le^x antigens, which are not unique component for tumor cell membranes, can be found in normal cells. However, with oncogenic transformation these nonspecific structures may be organized in such a way that they are recognized by the host immune cells as well as the antibodies, becoming tumor-associated antigens. The antibodies against tumor-associated antigens have a relatively broad spectrum of tumor-specificity to various cancer cell lines including humans. Therefore, the immunoliposomes sensitive to the tumor-associated antigens may be more broadly applicable to various human cancers.

We are now involved in *in vitro* and *in vivo* tests to examine the therapeutic efficacy of doxorubicin encapsulated in the immunoliposomes, compared to free DOX and conventional liposomes containing DOX. We are also preparing long-circulating immunoliposomes containing DOX to achieve better *in vivo* targetability to tumor cells. This type of immunoliposome formulations will be experimentally and clinically useful for the delivery of bioactive materials, such as chemical drugs, protein, DNA, etc.

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