AAPS PharmSciTech 2006; 7 (2) Article 32 (http://www.aapspharmscitech.org).

A Tumor Vasculature Targeted Liposome Delivery System for Combretastatin A4: Design, Characterization, and In Vitro Evaluation

Submitted: September 9, 2005; Accepted: January 30, 2006; Published: April 7, 2006

Ramakrishna Nallamothu,[1] George C. Wood,[1] Christopher B. Pattillo,[2] Robert C. Scott,[2] Mohammad F. Kiani,[2,3] Bob M. Moore,[4] and Laura A. Thoma[1]

[1]Parenteral Medications Laboratories, Department of Pharmaceutical Sciences, University of Tennessee Health Sciences Center, Memphis, TN
[2]Department of Mechanical Engineering, Temple University, Philadelphia PA
[3]Department of Radiation Oncology, Temple University, Philadelphia, PA
[4]Department of Pharmaceutical Sciences, University of Tennessee Health Sciences Center, Memphis, TN

ABSTRACT

The objective of this study was to develop an efficient tumor vasculature targeted liposome delivery system for combretastatin A4, a novel antivascular agent. Liposomes composed of hydrogenated soybean phosphatidylcholine (HSPC), cholesterol, distearoyl phosphoethanolamine-polyethylene-glycol-2000 conjugate (DSPE-PEG), and DSPE-PEG-maleimide were prepared by the lipid film hydration and extrusion process. Cyclic RGD (Arg-Gly-Asp) peptides with affinity for αvβ3-integrins expressed on tumor vascular endothelial cells were coupled to the distal end of PEG on the liposomes sterically stabilized with PEG (long circulating liposomes, LCL). The liposome delivery system was characterized in terms of size, lamellarity, ligand density, drug loading, and leakage properties. Targeting nature of the delivery system was evaluated in vitro using cultured human umbilical vein endothelial cells (HUVEC). Electron microscopic observations of the formulations revealed presence of small unilamellar liposomes of ~120 nm in diameter. High performance liquid chromatography determination of ligand coupling to the liposome surface indicated that more than 99% of the RGD peptides were reacted with maleimide groups on the liposome surface. Up to 3 mg/mL of stable liposomal combretastatin A4 loading was achieved with ~80% of this being entrapped within the liposomes. In the in vitro cell culture studies, targeted liposomes showed significantly higher binding to their target cells than nontargeted liposomes, presumably through specific interaction of the RGD with its receptors on the cell surface. It was concluded that the targeting properties of the prepared delivery system would potentially improve the therapeutic benefits of combretastatin A4 compared with nontargeted liposomes or solution dosage forms.

Corresponding Author: Ramakrishna Nallamothu, 26 S Dunlap St, Room 214, Memphis, TN 38163. Tel: (901) 448-4637; Fax: (901) 448-6092; E-mail: rnallamo@utmem.edu

KEYWORDS: targeted liposome delivery system, combretastatin A4, tumor vasculature targeting, liposome characterization.

INTRODUCTION

Liposomes are well-recognized drug delivery vehicles. They have been shown to enhance the therapeutic activity of several anticancer drugs.[1] Appropriately designed liposomes (long circulating liposomes, LCL) have the ability to passively accumulate in tumor tissues, which are known to exhibit the, so-called, leaky vasculature.[2] This passive accumulation can be further enhanced by actively targeting LCL to these tumor areas. Targeting of such liposomes, achieved by coupling ligands to the vesicle surface, could potentially reduce the extent of nonspecific toxicity associated with drug. Ligand-targeted liposomal anticancer drugs have been shown to have increased binding, improved cytotoxicity, and in many cases improved therapeutic efficacy, compared with nontargeted liposomes.[3,4]

Combretastatin A4 is a novel antivascular agent. It has shown high anticancer activity by inducing irreversible vascular shutdown in solid tumors.[5] A phosphate salt form of this drug is currently in phase 2 clinical trials as an intravenous (IV) infusion dosage form. Despite its anticancer potential, the drug has several undesirable side effects in many normal tissues.[6] These problems can be alleviated by targeting the drug specifically to the solid tumor vasculature. It has been shown that certain cell adhesion molecules such as $\alpha_v\beta_3$ integrin receptors are overexpressed on actively proliferating endothelium of the tumor vasculature.[7,8] These surface markers discriminate tumor endothelial cells from the normal endothelial cells and can be used as a target for antivascular drug delivery. Peptides with Arg-Gly-Asp (RGD) amino acid sequence constrained in a cyclic framework were shown to bind to these $\alpha_v\beta_3$ integrin receptors.[9] Using these facts, we designed a targeted liposome delivery system for combretastatin A4 with cyclic RGD peptides as targeting ligands (Figure 1). We have already shown that targeting of combretastatin A4 to irradiated tumors using this delivery system results in significant tumor

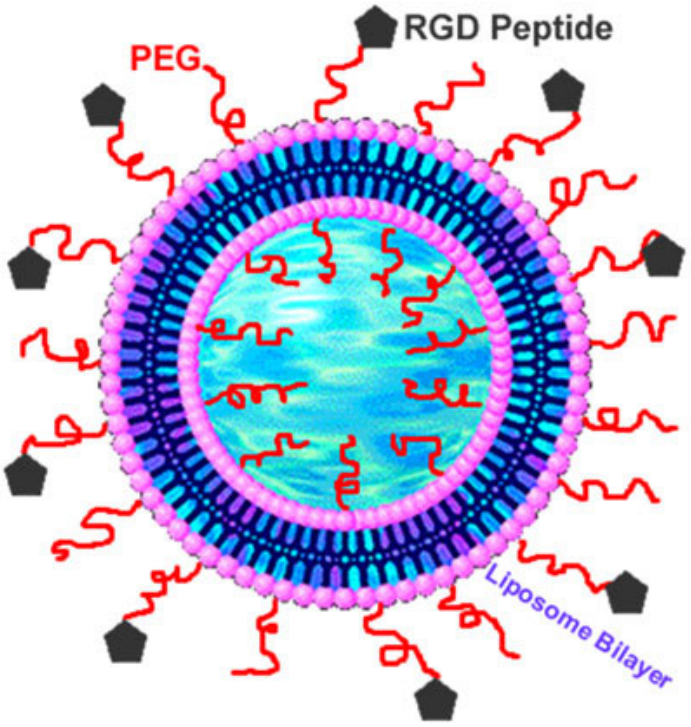

Figure 1. Schematic representation of the targeted liposome delivery system. Cyclic RGD peptides coupled to the distal end of maleimide-PEG-DSPE in the liposome bilayer of PEG-grafted LCL.

growth delay.[10] In this study, the design features of the system, its characterization and in vitro evaluation are described. The delivery system was characterized in terms of size, ligand density, drug loading, and leakage. Association of this targeted delivery system with the target endothelial cells was evaluated in vitro using cell culture studies.

Materials and Methods

Materials

Hydrogenated soybean phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PEG (2000)] conjugate (DSPE-PEG), and DSPE-PEG-maleimide were obtained from Northern Lipids Inc (Vancouver, BC, Canada). Cholesterol was obtained from Sigma, (St Louis, MO), and the RGD peptide (cyclo [Arg-Gly-Asp-D-Phe-Cys]) was custom synthesized (purity 99.9%) by Peptides International (Louisville, KY). These chemicals were used as received. Combretastatin A4 was synthesized according to the methods of Pettit et al[11] and is 97.8% pure as determined by high performance liquid chromatography (HPLC, Nova-Pak C_{18}-column, methanol:water [50:50] mobile phase, 0.8 mL/min flow rate, and 295 nm UV detection). All other chemicals and solvents used were of analytical grade.

Preparation of Long Circulating Liposomes

Long circulating liposomes were composed of HSPC, cholesterol, and DSPE-PEG in the molar ratio of 65:30:5, respectively. In case of radiolabeled liposomes cholesteryl [4-^{14}C] oleate (American Radiolabeled Chemicals Inc, St Louis, MO) was included in the formulation at $1{\cdot}48\times10^4$/μmol of lipid. Accurately weighed amounts of lipids (325 μmol HSPC, 150 μmol cholesterol, and 25 μmol DSPE-PEG) and drug (100 μmol) were dissolved in chloroform:methanol (9:1 vol/vol) in a round-bottom flask. After mixing, solvent was evaporated under reduced pressure and constant rotation (Rotovapor R-200, Buchi, Flawil, Switzerland) to form a thin lipid film. The lipid film was then hydrated with 50 mM HEPES/150 mM NaCl-buffer pH 6.5 (5 mL) at 62°C for 2 hours to form large multilamellar vesicles (MLV) at 100 mM total lipid concentration. The resulting MLV were then sized by repeated extrusion (Lipex extruder, Northern Lipids) through polycarbonate membranes (Nucleopore, Whatman, NJ) of gradually decreasing pore size (0.8, 0.4, 0.2, and 0.1 μm) to prepare small unilamellar liposomes of ~100 nm in diameter.[12] Extrusions were performed in a 10-mL size thermobarrel extruder at 62°C. After extrusion, liposomes were stored at 4°C until used in subsequent experiments.

Preparation of Targeted Liposomes

For preparation of targeted liposomes, RGD recognition motifs constrained in a cyclic conformation were coupled to the distal end of PEG chains on the long circulating liposomes. To enable this ligand coupling, a part of (2 mol%) DSPE-PEG in the long circulating liposome formulation was replaced with DSPE-PEG-maleimide functional lipid. Total lipid concentration of the liposomal dispersion used for the coupling reaction was 100 mM. Cyclo(Arg-Gly-Asp-D-Phe-Cys) peptides dissolved in 50 mM HEPES buffer, pH 6.5 at 2.7 μmol/mL concentration (0.1 mL), were reacted with LCL (4 mL) with maleimide functional groups on the distal end of PEG chains at pH 6.5 and a molar ratio of 1:30 (RGD:maleimide) for 12 hours at room temperature (25°C). A schematic representation of the coupling reaction is given in Figure 2.

HPLC Determination of RGD Coupling to the Liposomes

Attachment of RGD peptides to the liposome surface was ascertained indirectly by determining noncoupled peptide fraction using HPLC. A C_{18}-column (Nova-Pak 3.9 × 150 mm, Waters, Milford, MA) was used with a mobile phase of 0.05% trifluoroacetic acid in water (eluant A) and 0.05% trifluoroacetic acid in acetonitrile (eluant B). The eluant gradient was set from 10% to 60% B in 50 minutes and subsequently back to 10% B over 5 minutes. Flow rate was

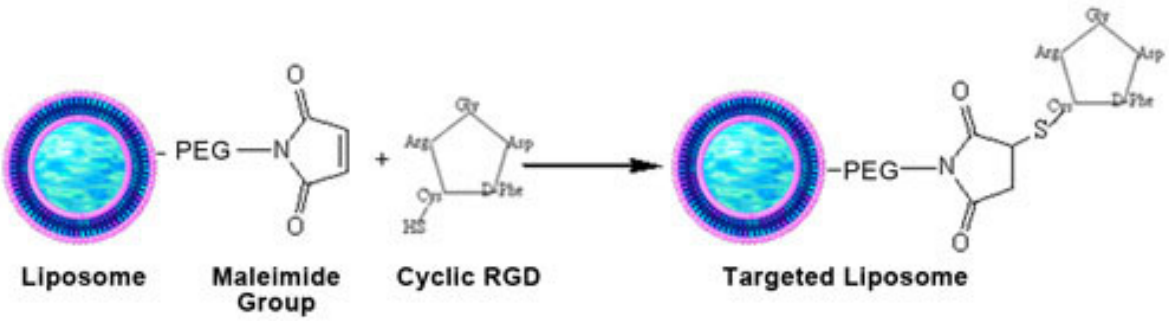

Figure 2. Schematic representation of the coupling reaction between maleimide functional group at the distal end of PEG chain on the LCL and thiol group in the cyclic RGD peptide.

1 mL/min and between measurements the column was equilibrated for 10 minutes with 90% A and 10% B. The uncoupled peptide was detected by measuring absorbance at 214 nm.

Estimation of Drug Entrapment in Liposome Formulation

Total and free combretastatin A4 in the liposome formulations were determined using HPLC analysis. Total drug was determined after ethanol extraction. An aliquot of the liposome dispersion (50 μL) was diluted to 2 mL with ethanol to release liposome-encapsulated combretastatin A4. Total combretastatin A4 in this clear ethanol extract was determined using HPLC, with a C_{18}-column (Nova-Pak 3.9 × 150 mm column, Waters) and methanol:water (50:50) as mobile phase. Flow rate of 0.8 mL/min and UV detection at 295 nm were used.

Free combretastatin A4 was separated from the liposome encapsulated part using a Centricon centrifugal filter device (Centricon 10, MWCO 10 kd, Millipore, Bedford, MA). An aliquot of the liposome dispersion (100 μL) was diluted to 1 mL with hydration buffer (50 mM HEPES/150 mM NaCl-buffer pH 6.5), and this sample was transferred to the centrifugal filter device. The sample was centrifuged at 10 000 rpm for 15 minutes in a fixed-angle centrifuge. Free combretastatin A4 in the filtrate was then determined using HPLC. Dilution factors were taken into consideration for calculation of total and free drug. Subtraction of free drug from the total drug gave the amount of liposome-entrapped drug. Drug estimations were done in triplicate, and the values were reported as mean ± SEM.

Visualization and Size Measurements

Large MLV, before the extrusion process, were visualized using a light microscope (Olympus, CKX41, Tokyo, Japan). Final liposomes were visualized under electron microscope by negative staining technique. A diluted liposome sample was adsorbed onto a formavar- and carbon-coated copper grid, stained with 2% uranyl acetate (pH 7.0) and observed with a JEM1200EX electron microscope (JEOL, Tokyo, Japan) at ×50 000 magnification. Size and size distribution profiles of liposomes were monitored by dynamic light scattering method using the Malvern Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK).

In Vitro Leakage Studies

Liposome encapsulation stability of combretastatin A4 was monitored in vitro, by dialyzing samples for 48 hours against 700 volumes of reverse osmosis water maintained at 37°C. A 0.5-mL aliquot of the liposome dispersion was placed in a presoaked Pierce dialysis cassette (Slide-A-Lyzer, MWCO 10 kd, Millipore), which was then placed in a beaker containing 350 mL of release medium pre-equilibrated to 37°C. The dialysis cassette was rotated at 100 rpm. The volume of release medium was selected based on careful consideration of sink conditions and sensitivity of the analytical method. At different time points, 0.5-mL samples were taken from the release medium and replaced with an equal volume of fresh release medium. Samples were analyzed for the released drug, combretastatin A4, using the HPLC method of analysis. From the total drug concentration of the liposome formulation, percentage released at each time point was calculated. Results are reported as mean ± SEM (n = 3).

In Vitro Endothelial Cell Binding Studies

To investigate the extent of association of the liposomes with the target vascular endothelial cells, an in vitro cell culture system using human umbilical vein endothelial cells (HUVEC) was developed. The levels of cell-associated liposomes were monitored by following the liposome bilayer incorporated cholesteryl[4-^{14}C] oleate, a label that has been proven to be nonexchangeable.[13]

HUVEC (Glycotech, Gaithersburg, MD) were cultured in medium 199 (Cambrex, Baltimore, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL

heparin, 50 μg/mL endothelial mitogen (Biomedical technologies, Stoughton, MA), and penicillin/streptomycin.[14] Cells were maintained in 75-cm^2 plastic culture flasks in the above medium at 37°C in a humidified atmosphere containing 5% CO_2 in air and subcultured by transferring small amounts of cell suspension into fresh flasks with medium. Cells were subcultured at 80% confluence.

For liposome binding studies, 1×10^5 cells/100 μL were plated in 24-well culture plates coated with 1.0% gelatin and left to adhere and grow for 2 days. Various formulations of cholesteryl[4-^{14}C] oleate labeled liposomes, with or without coupled ligands, were added (100 μL) to each well and maintained at 37°C in the humidified atmosphere containing 5% CO_2 in air for 4 hours in the absence of FBS. In competitive experiments, liposome binding was conducted in the presence of excess free RGD (50 mg/mL) that was added 30 minutes before the addition of liposomes. Incubations were terminated by removing the incubation medium followed by thorough washing with ice cold PBS (pH 7.4) 4 times. Cells were harvested with trypsin treatment (0.05%) for 2 minutes at 37°C, centrifugation, and a subsequent wash of the cell pellet with PBS. Trypsin removable label in the combined supernatants and trypsin nonremovable label in the cell pellet, after lysing the cells with 0.5 M NaOH and solubilizing with Soluene 350 (PerkinElmer, Boston, MA), were determined by scintillation counting using a Beckman LS-6800 counter (Beckman Coulter Inc, Fullerton, CA). Total cell-associated label was determined by combining radioactivity in the supernatant (removable label) and in the cell pellet (nonremovable label). All studies were performed in triplicate, and the results are reported as mean ± SEM.

Results and Discussion

Formulation Design Features

An effective liposomal targeted delivery system should satisfy several requirements. It should incorporate high drug loading with stable encapsulation and should possess good physical and chemical stability during shelf life. In addition, targeted liposomes must survive in the systemic circulation long enough to reach, find, and bind to their target. Drug should not be released during this in vivo transit. Association of ligand-bearing liposomes with target cells in vivo occurs more effectively when the former circulate in the blood in a stable form (so that liposomes can retain much of their ligands and drug content) and for periods of time long enough to ensure sufficient vesicle exposure to the cells.[15] Once at the target site, the delivery system should release the drug at rates that will result in improved therapeutic effects over that achievable for the free drug. The elements of the targeted delivery system were chosen to maximally satisfy these requirements.

The targeted liposome delivery system is composed of 4 different lipid components: HSPC, cholesterol, DSPE-PEG, and DSPE-PEG-maleimide. HSPC is the principal component of the lipid bilayer. Being a saturated lipid, HSPC is relatively stable for chemical degradation. HSPC also minimizes premature drug leakage from the liposomes during both storage on shelves and in vivo transit, before they reach target site. It is known that lipid bilayers become more permeable near and above their phase transition temperature (T_m) because of their transformation to liquid crystalline state. Because of its high T_m of 54°C, HSPC would keep the bilayer hydrocarbon core in the solid (gel) state at both room (shelf) and body temperatures. Liposome bilayers with these characteristics are more resistant to leakage than those composed of unsaturated phospholipids, whose bilayer core tends to be fluidy at body temperature. HSPC also minimizes batch-to-batch variability in the lipid composition and is free from immunogenic reactions, unlike the commonly used egg phosphatidyl choline. Cholesterol is included in the formulation to give further rigidity to the bilayer.[16] Presence of cholesterol in liposome formulation was also shown to enhance retention of entrapped solutes and reduce serum-induced instability caused by binding of serum proteins to liposome membrane.[17] Through these effects cholesterol improves both in vitro and in vivo stability of the liposomes. PEG-conjugated lipids are incorporated to enhance the circulation longevity of the liposomes through steric barrier to opsonization and mononuclear phagocytic system (MPS) uptake.[18] The DSPE portion of the conjugated lipids, being part of the lipid bilayer, increases loading of the lipophilic drug combretastatin A4 and contributes to the bilayer stability. Maleimide functionalized lipid allows conjugation of the RGD ligands to the liposome surface.

Ligand Coupling

Several methods for binding of ligands to liposomes were described.[19] One of the most useful and efficient coupling chemistries involved conjugation of thiolated ligands to liposomes grafted with maleimide groups. The reaction between maleimide and thiol groups is rapid and proceeds close to completion.[20] Furthermore, it takes place at close to neutral pH, at ambient temperature, and even when relatively low concentrations of the reactants are present. So, it is particularly suitable for interlinking ligands to liposomes. Therefore, we used this coupling reaction to conjugate thiol-containing RGD peptides to the distal end of PEG chains on the LCL through maleimide functional groups (Figure 2). This is a Michael addition type reaction and results in covalent attachment of the RGD peptide onto the liposome surface through thio-ether bond. The resulting thio-ether bond is stable under physiological conditions. Hence, the ligand will not dissociate from the liposome in

the systemic circulation,[19] meeting another important requirement for a stable targeted delivery system.

Another significant design feature of our targeted delivery system is attachment of targeting ligands at the distal end of PEG chains on the sterically stabilized LCL. A targeted delivery system made by attaching ligands at PEG terminus on sterically stabilized liposome is more effective than one made by attaching ligands directly onto the surface of PEG-containing liposome. In the latter case, PEG chains were shown to interfere with both the coupling of ligands to the lipid bilayer and the interaction of these ligands with the intended biological targets.[19] To avoid this interference, in our delivery system, ligands were attached to the distal end of PEG chains. This ligand coupling to the PEG terminus does not cause any interference with the binding of ligands to their respective recognition molecules.[19,21] Binding sites of liposome-conjugated RGD peptides will remain accessible for unperturbed interaction with the target molecule on the surface of the target cells. Furthermore, the PEG coating of the liposome surface will continue to retard unwanted opsonization of the liposomes, leading to their extended circulation half-lives.[21] Thus, this method of making a targeted delivery system combines long circulation times with effective target binding.

HPLC Determination of RGD Coupling to the Liposomes

An HPLC method was developed to determine the extent of coupling of the RGD peptide to the liposomes. The method involved estimating the amount of free RGD left in the formulation after the coupling reaction. Standard RGD dissolved in the mobile phase eluted at ~14-minute retention time as shown in Figure 3A. This peak was monitored for the estimation of free RGD in the final liposome formulations. The liposome formulation sample following the coupling step was injected to determine the amount of free RGD left unreacted. As shown in Figure 3B, there was no significant peak for the free RGD around 14 minutes, indicating that there was no considerable amount of free RGD left unreacted in the formulation. Therefore, almost all the RGD peptide added to the formulation (>99%, based on detection limit of the assay) had been coupled to the liposomes. As we know, RGD coupled to the liposomes would give a different retention time than the free RGD. To test the sensitivity of the HPLC method to detect free RGD in the presence of liposomes, an excess free RGD was added to the final targeted liposome formulation sample (positive control) and analyzed using the same HPLC method. As shown in Figure 3C, in this case we obtained the peak for the free RGD at 13.818-minute retention time. It clearly shows that the HPLC method is sensitive to detect free RGD in the presence of liposomes. Therefore, the absence of peak for the free RGD in the final

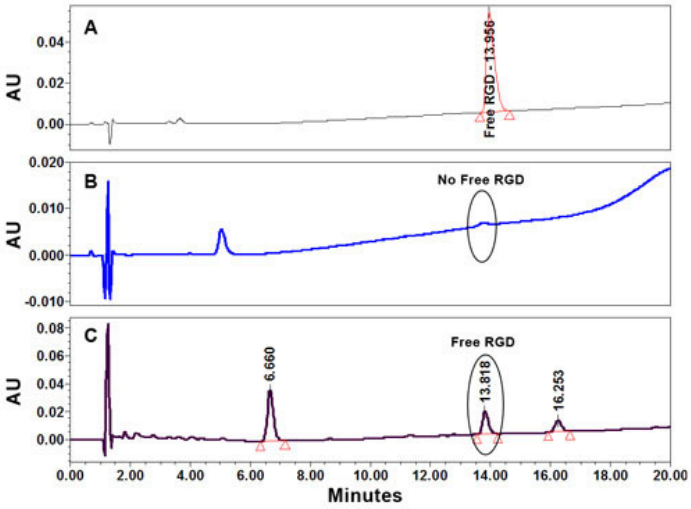

Figure 3. HPLC confirmation of RGD coupling to the liposomes. (A) Standard free RGD eluted with a retention time of ~14 minutes; (B) the liposome formulation sample following the coupling step showed no significant peak for the free RGD around 14 minutes; and (C) excess free RGD added to the liposome formulation sample (positive control) gave the peak for the free RGD.

liposome sample (Figure 3B) indicates that the coupling reaction was complete. Because of the high reactivity of the maleimido group, we obtained near 100% coupling efficiencies in line with those reported in the literature.[19]

From the amount of RGD peptide used and the phospholipid concentration of the formulation, we calculated that there were ~124 RGD peptides present on each liposome. These calculations were based on the assumption that 144 000 phospholipid molecules form one liposome vesicle of 120 nm.[22] The number of phospholipid molecules was estimated from the experimentally determined phospholipid concentration. Phospholipid concentrations of the liposome preparations were determined by a phosphate assay after perchloric acid destruction.[23] Total liposomal lipid concentrations were calculated, taking into account the amount of cholesterol in the liposome preparation.

Sizing and Visualization

Size of the liposomes affects their circulation half-life ($T_{1/2}$). As the size increases, the $T_{1/2}$ decreases. Optimum size for long circulation was found to be 114.3 nm.[24] In order to prepare liposomes of this optimum size reproducibly, we used the extrusion process. The extrusion process used to prepare the liposomes had been shown to produce primarily small unilamellar liposomes.[12] It is a gentler method of reducing the size of liposomes and produces a relatively monodisperse vesicle population of controlled average size without introduction of contaminants. The apparatus used

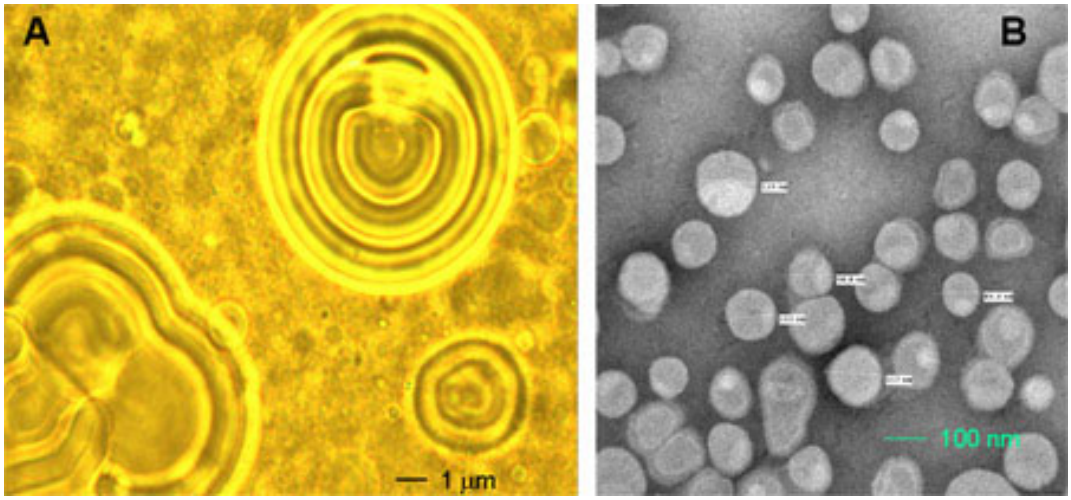

Figure 4. (A) Large multilamellar vesicles before extrusion process visualized under light microscope at original magnification ×100; (B) transmission electron micrographs of the final targeted liposomes visualized by the negative staining technique at original magnification ×50 000.

for extrusion is the same as that employed for other membrane pressure filtration applications. The process can be scaled up simply by increasing the area of the membrane used.

As the multilamellar liposomes are extruded through the membranes of decreasing pore size repeatedly, the liposome suspension becomes progressively more unilamellar in character on each passage, with the vesicles still maintaining a size distribution around the pore size of the membrane. An almost completely unilamellar population can be produced after 5 to 10 repeated extrusions through 2 stacked membranes.[12] Figure 4 shows light and electron photomicrographs of the liposomes before and after the extrusion step, respectively. Unextruded liposomes in the right panel (Figure 4A) are, as expected, nonuniform and show several layers that are characteristic of multilamellar vesicles. In contrast, the vesicles in the left panel (Figure 4B) are uniform in size, almost spherical in shape, and range in diameter from 80 to 130 nm. The vast majority of the vesicles fall in the size range of 100 to 120 nm, the mean diameter being 114.16 nm. However, the negative staining electron microscopy revealed little difference in the morphology of the RGD-coupled liposomes compared with nontargeted liposomes. These 2 photomicrographs clearly demonstrate that the extrusion process made the liposome preparation uniform in size and yielded small unilamellar liposomes that are necessary for prolonged circulation times.

The exact size and size distribution of the liposome preparations were also determined using the standard method of dynamic light scattering with Malvern Zetasizer Nano and are shown in Figure 5. The diameter of the final liposomes was in the range of 123.8 ± 41.2 nm. The system reported a polydispersity index (PDI) as a measure of particle size distribution. The PDI ranged from 0.032 to 0.125, indicating a uniform monodisperse system. Presence of RGD peptide on the liposome surface slightly increased the size compared with liposomes without ligands. LCL without ligands were in the size range of 106.3 ± 27.5 nm.

Combretastatin A4 Loading Into the Liposomes

Relatively lipophilic combretastatin A4 was incorporated into the liposome bilayer by dissolving it in the organic phase along with the lipid components. Effect of lipid concentration, drug-to-lipid ratio, cholesterol, and DSPE-PEG content in the formulation on the liposomal loading of combretastatin A4 was studied. Table 1 summarizes the influence of these formulation variables on combretastatin A4 liposomal loading. By changing the formulation variables, up to 3 mg/mL of stable liposomal combretastatin A4 loading was obtained with ~80% of this being entrapped within the liposomes. Thirty percent cholesterol and 5% DSPE-PEG content in the formulation were found to be optimum for maximum combretastatin A4 loading and its minimal leakage.

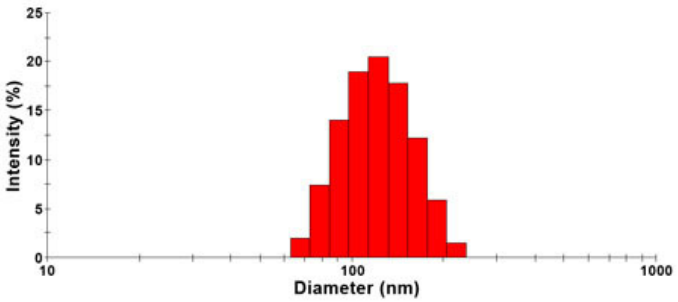

Figure 5. Size and size distribution profile of the targeted liposomes using light scattering method. Average diameter, 123.8 nm and PDI, 0.122.

Table 1. Influence of Formulation Variables on Liposomal Loading of Combretastatin A4*

Formulation Code	Bilayer Lipid Composition HSPC:Chol: DSPE-PEG	Lipid Concentration in the Formulation (mM)	Drug-to-Lipid Ratio (molar)	Total Drug Concentration Achieved in the Formulation (mg/mL) (mean ± SEM)	Percentage Entrapped Drug (mean ± SEM)
A	65:30:5	60	20:100	0.758 ± 0.214	71.114 ± 3.216
B	65:30:5	100	10:100	1.052 ± 0.125	88.023 ± 4.108
C	65:30:5	100	20:100	1.549 ± 0.143	84.258 ± 3.268
D	95:0:5	100	20:100	5.683 ± 0.207	95.742 ± 2.143
E	50:45:5	100	20:100	0.769 ± 0.228	68.302 ± 3.649
F	62.5:30:7.5	100	20:100	1.813 ± 0.164	89.355 ± 2.115
G	65:30:5	200	20:100	2.110 ± 0.213	92.846 ± 2.062
H	65:30:5	200	40:100	2.996 ± 0.108	80.952 ± 3.139

*HSPC indicates hydrogenated soybean phosphatidylcholine; Chol, cholesterol; DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol)2000] conjugate; and SEM, standard error of the mean (n = 3).

In Vitro Leakage Properties

Stable encapsulation is essential for successful targeting of liposomes. Drug released from liposomes en route to the target site may not contribute effectively to a therapeutic effect at the intended disease sites. Stability of combretastatin A4 liposomal encapsulation was tested using the in vitro release methodology.

Figure 6 shows combretastatin A4 leakage from the targeted liposomes and LCL in comparison to that of ethanol solution. As ethanol solution contains combretastatin A4 in free form, it released the drug quickly (71.25% ± 1.1% in 3 hours), validating the in vitro release methodology. In agreement with the previous studies,[16] LCL with no cholesterol in them (formulation D) leaked out their contents rapidly. As the cholesterol content of the formulation increased, drug leakage decreased. At 30% cholesterol level there was no significant leakage within 48 hours (formulation C). However, at still higher cholesterol levels (45%) not only did drug loading decrease (Table1) but also leakage increased. Based on these results, 30% cholesterol content was selected into the formulation to obtain optimum loading and stable encapsulation of combretastatin A4. The amount of DSPE-PEG in the formulation influenced drug loading as well as the bilayer stability. Although 7.5% DSPE-PEG in the formulation (formulation F) increased drug loading, it also increased drug leakage compared with formulation C with 5% DSPE-PEG. Therefore, 5% DSPE-PEG was selected into the formulation, which would give the required steric protection without compromising the bilayer integrity.

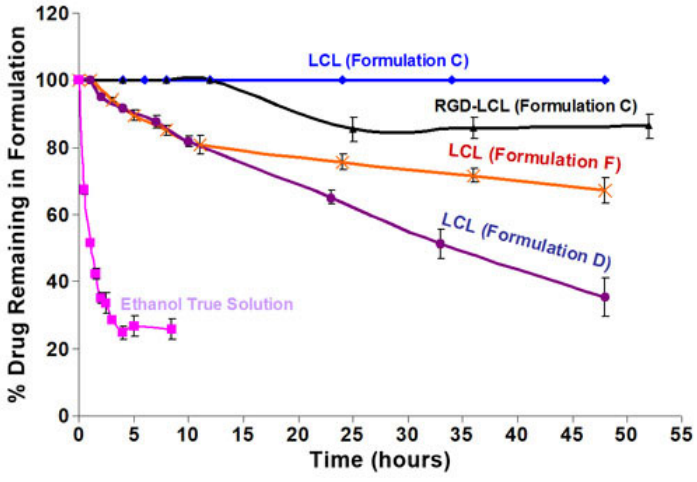

Figure 6. In vitro leakage of combretastatin A4 from the targeted liposomes (RGD-LCL) in comparison with that of nontargeted liposomes (LCL) of various bilayer compositions and ethanol true solution.

RGD-coupled targeted liposomes released 13.72% ± 4.1% of the loaded drug within 52 hours, whereas the LCL with the same bilayer composition (without ligands) released no significant amount of the drug within this period. These results indicate that ligand attachment caused some kind of membrane destabilization in the targeted liposomes leading to faster drug leakage. Bredehorst et al, in their earlier study[25] have also reported that binding of antibody fragments to the liposome surface caused faster release of the entrapped drug. These authors attributed this faster leakage to possible swelling and rupture of the liposome membrane caused by ligand attachment. Studies to further optimize the targeted liposome formulation to minimize this premature drug leakage are underway in our laboratory.

In Vitro Target Cell Binding Evaluation

As specific association of liposomes with their target cells is a necessary requirement for target specific drug delivery, we tested whether our targeted liposomes specifically bound to the target cells in vitro. To evaluate the extent of association of the prepared delivery system with the target vascular endothelial cells, an in vitro HUVEC culture system was developed. Tumor vascular endothelial cells were shown to express $\alpha_v\beta_3$ integrin receptors on their luminal surface. This expression is further increased during angiogenesis

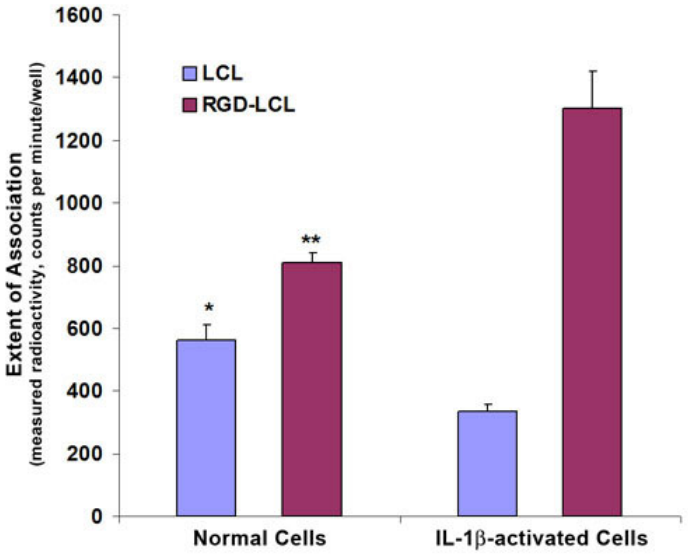

Figure 7. Extent of association of the cholesteryl[4-^{14}C] oleate labeled targeted (RGD-LCL) and nontargeted liposomes (LCL) with normal and IL-1β-activated HUVEC monolayers in vitro. Levels *, ** are significantly different ($P < .05$).

or in response to inflammatory stimuli. It is this high expression of $\alpha_v\beta_3$ that we want to use to target our drug delivery system toward tumor vasculature. Cultured HUVEC activated with pro-inflammatory stimuli such as cytokine interleukin 1β (IL-1β) have been known to express increasing levels of many cell adhesion molecules, making it one of the most useful in vitro model systems for cell biological studies.[26] These cells also express $\alpha_v\beta_3$ as a major cell surface molecule.[27] Furthermore, similar to in vivo tumor vascular endothelial cells, these cultured HUVEC also upregulate $\alpha_v\beta_3$ expression in response to inflammatory stimuli.[28] Using this rationale, we have compared association of our targeted liposomes with normal HUVEC monolayers and those activated with IL-1β.

In Figure 7, the extent of association of cholesteryl[4-^{14}C] oleate labeled liposomes with normal and IL-1β activated cells is shown. Association of targeted liposomes (RGD-LCL) was significantly higher ($P < .05$) than that of nontargeted liposomes (LCL) with both normal and IL-1β-activated cells. The difference between targeted and nontargeted liposome interaction was wider with IL-1β activated cells. While targeted liposomes associated with normal cells ~1.5 times that of LCL, their association with IL-1β-activated cells was as high as 4 times that of LCL. This superior association of targeted liposomes to these cells could be to the result of specific interaction of RGD ligand with its recognition molecules. Increased expression of specific receptors for the RGD, upon inflammatory stimuli with IL-1β, increased association of targeted liposomes. As nonactivated (normal) cells express these receptors only to a lesser extent, association of targeted liposomes with them was less than that of activated cells. LCL without any ligands on them, on the other hand, would only associate through simple nonspecific adsorption. But this nonspecific adsorption was not as substantial as that of specific ligand-receptor interaction of targeted liposomes. Hence, LCL associated with HUVEC only to a lesser extent than targeted liposomes. Of interest, this nonspecific interaction of LCL decreased upon IL-1β activation. Cell surface with many upregulated receptors, as is the case with IL-1β activation, may not be conducive to nonspecific interaction of LCL.

Total radiolabeled liposomes associated with the cells reflect a combination of binding to the cell surface as well as internalization. In Figure 8, total association of the cholesteryl[4-^{14}C] oleate labeled liposomes with the HUVEC monolayers was segmented into trypsin removable and nonremovable fractions in each case. Trypsin removable label was obtained by trypsinization and centrifugation of the lifted cells and measuring the liposomal radioactivity in the supernatant. This fraction represents the liposomes that are loosely bound to the cell surface.[29] These loosely bound liposomes may have associated with cells either by nonspecific interactions or by weak ligand-receptor interactions. This liposome fraction eluted from the cells with trypsin treatment. Both targeted and nontargeted liposomes have a substantial portion of their total association (~60%) as trypsin removable label. Trypsin nonremovable label, the label that remained with cell pellet, then represents the liposomes that are strongly bound to cell surface or most probably liposomes that are taken up by the cells.[29] As shown in Figure 8, higher association of targeted liposomes with normal HUVEC monolayers was

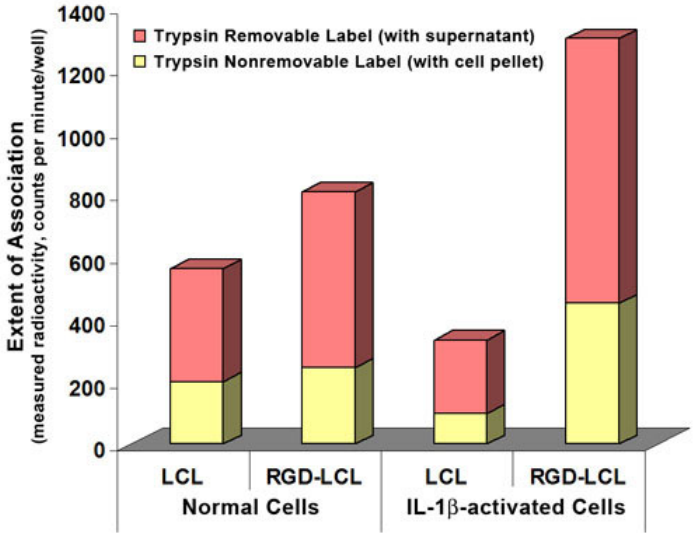

Figure 8. Extent of association of the cholesteryl[4-^{14}C] oleate labeled targeted (RGD-LCL) and nontargeted liposomes (LCL) with normal and IL-1β-activated HUVEC monolayers in vitro, segmented into trypsin removable fraction (binding) and trypsin nonremovable fraction (uptake).

mainly owing to the higher binding. Uptake was not significantly different from that of LCL. The higher association of the targeted liposomes with the IL-1β-activated cells was owing to both increased binding as well as uptake. Activation of cells led to increased expression of receptors for the RGD ligands, which increased specific ligand-mediated interaction of liposomes with cells. Increased interaction of liposomes with cells through this ligand-mediated binding also led to their increased uptake, presumably through receptor-mediated endocytosis.[30] This finding suggests that liposome binding to cells is the factor determining the extent of uptake. As the extent of binding increased, number of liposomes taken up by the cells also increased. Similar results were reported by Kirpotin et al[31] with anti-HER2 immunoliposomes.

To further prove that the higher association of the targeted liposomes with the in vitro HUVEC monolayers was mainly through the ligand-mediated interaction, we also studied this association in the presence of excess free RGD. While the presence of excess free RGD did not make any difference with the mostly nonspecific interaction of LCL, the extent of association of the targeted liposomes decreased to the level of LCL (Figure 9). This finding shows that targeted liposomes associated with HUVEC mainly through the specific interaction of RGD with its receptors on the cell surface ($\alpha_v\beta_3$). Saturation of these receptor sites with excess free RGD decreased the targeted liposome association to the level of simple nonspecific interaction. This is the conclusive evidence that our targeted liposomes associated with cells to a higher degree mainly through RGD-mediated specific interaction.

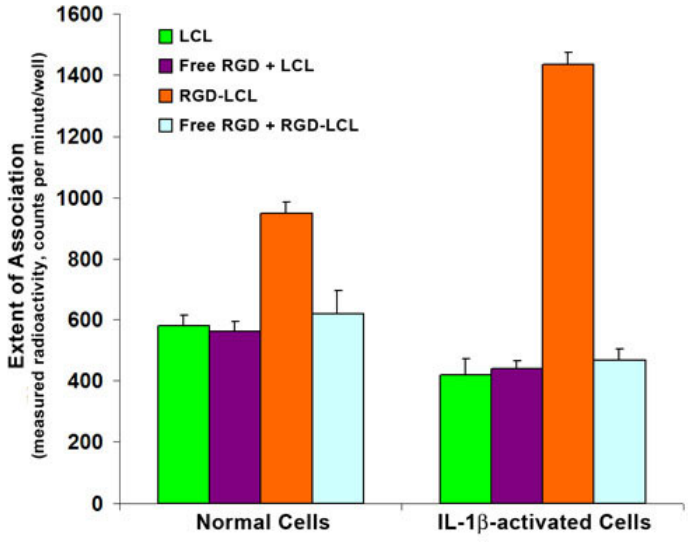

Figure 9. Extent of association of the cholesteryl[4-^{14}C] oleate labeled targeted (RGD-LCL) and nontargeted liposomes (LCL) with normal and IL-1β-activated HUVEC monolayers in vitro in the presence and absence of excess competing free RGD.

Thus, these in vitro studies demonstrated that our targeted liposomes associate to a greater extent with the target cells than nontargeted liposomes. This increased target binding in vitro can lead to increased cytotoxicity in vivo and consequently to improved therapeutic effects.[3] It was previously demonstrated that LCL of small size (~100 nm in diameter) and rigid lipid composition accumulate to a greater extent in solid tumor.[2] In this study, we have prepared targeted liposomes of ~120 nm in diameter with rigid lipid composition and potentially long circulating properties. As defects in the capillary endothelium of the tumor vasculature are typically in the size range of 200 to 600 nm,[32] we expect our liposomes to effectively accumulate in and around tumor vasculature. This accumulation will be further enhanced by the targeting nature of our liposomes. It should also be noted that the target sites for our delivery system are located at a relatively readily accessible site (ie, the vascular endothelial surface). As mentioned earlier, we have already demonstrated that targeting of combretastatin A4 to irradiated tumors using our targeted delivery system results in effective tumor growth delay.[10]

Conclusions

A tumor vasculature targeted liposome delivery system was developed for the novel antivascular drug, combretastatin A4. These targeted liposomes showed significantly higher binding to their target cells than nontargeted liposomes in the in vitro model system examined. This targeting effect may increase the anticancer activity of the drug and potentially improve its therapeutic benefits compared with nontargeted liposomal or solution dosage forms.

Acknowledgement

Mohammad F. Kiani is an established investigator of the American Heart Association and is supported by a grant from the U.S. Department of Defense Breast Cancer Research Program.

References

1. Lasic DD, Papahadjopoulos D. *Medical Applications of Liposomes*. Amsterdam, The Netherlands: Elsevier; 1998.

2. Forssen EA, Coulter DM, Proffitt RT. Selective in vivo localization of daunorubicin small unilamellar vesicles in solid tumors. *Cancer Res.* 1992;52:3255–3261.

3. Park JW, Hong K, Kirpotin DB, Meyer O, Papahadjopoulos D, Benz CC. Anti-HER2 immunoliposomes for targeted therapy of human tumors. *Cancer Lett.* 1997;118:153–160.

4. Stephenson SM, Low PS, Lee RJ. Folate receptor-mediated targeting of liposomal drugs to cancer cells. *Methods Enzymol.* 2004; 387:33–50.

5. Dark GG, Hill SA, Prise VE, Tozer GM, Pettit GR, Chaplin DJ. Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. *Cancer Res.* 1997;57:1829–1834.

6. Young SL, Chaplin DJ. Combretastatin A4 phosphate: background and current clinical status. *Expert Opin Investig Drugs.* 2004;13:1171–1182.

7. Hynes RO. A reevaluation of integrins as regulators of angiogenesis. *Nat Med.* 2002;8:918–921.

8. Kumar CC, Armstrong L, Yin Z, et al. Targeting integrins alpha v beta 3 and alpha v beta 5 for blocking tumor-induced angiogenesis. *Adv Exp Med Biol.* 2000;476:169–180.

9. Haubner R, Gratias R, Diefenbach B, Goodman SL, Jonczyk A, Kessler H. Structural and functional aspects of RGD-containing cyclic pentapeptides as highly potent and selective integrin alpha v beta 3 antagonists. *J Am Chem Soc.* 1996;118:7461–7472.

10. Pattillo CB, Sari-Sarraf F, Nallamothu R, Moore BM, Wood GC, Kiani MF. Targeting of the antivascular drug combretastatin to irradiated tumors results in tumor growth delay. *Pharm Res.* 2005;22:1117–1120.

11. Pettit GR, Singh SB, Boyd MR, et al. Antineoplastic agents. 291. Isolation and synthesis of combretastatins A-4, A-5, and A-6(1a). *J Med Chem.* 1995;38:1666–1672.

12. Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta.* 1985;812:55–65.

13. New RRC, Black CDV, Parker RJ, Puri A, Scherphof GL. Liposomes in biological systems. In: New RRC, ed. *Liposomes: A Practical Approach.* Oxford, UK: IRL Press Ltd; 1993:221–252.

14. Prabhakarpandian B, Goetz DJ, Swerlick RA, Chen X, Kiani MF. Expression and functional significance of E-selectin on cultured endothelial cells in response to ionizing radiation. *Microcirculation.* 2001;8:355–364.

15. Gabizon A, Chemla M, Tzemach D, Horowitz AT, Goren D. Liposome longevity and stability in circulation: effects on the in vivo delivery to tumors and therapeutic efficacy of encapsulated anthracyclines. *J Drug Target.* 1996;3:391–398.

16. Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *Biochem J.* 1980;186:591–598.

17. Semple SC, Chonn A, Cullis PR. Influence of cholesterol on the association of plasma proteins with liposomes. *Biochemistry.* 1996;35:2521–2525.

18. Torchilin VP, Omelyanenko VG, Papisov MI, et al. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim Biophys Acta.* 1994;1195:11–20.

19. Hansen CB, Kao GY, Moase EH, Zalipsky S, Allen TM. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim Biophys Acta.* 1995;1239:133–144.

20. Martin FJ, Papahadjopoulos D. Irreversible coupling of immunoglobulin fragments to preformed vesicles: an improved method for liposome targeting. *J Biol Chem.* 1982;257:286–288.

21. Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim Biophys Acta.* 1995;1237:99–108.

22. Maurer N, Fenske DB, Cullis PR. Developments in liposomal drug delivery systems. *Expert Opin Biol Ther.* 2001;1:923–947.

23. Rouser G, Fkeischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* 1970;5:494–496.

24. Kawahara K, Sekiguchi A, Kiyoki E, et al. Effect of TRX-liposomes size on their prolonged circulation in rats. *Chem Pharm Bull (Tokyo).* 2003;51:336–338.

25. Bredehorst R, Ligler FS, Kusterbeck AW, Chang EL, Gaber BP, Vogel CW. Effect of covalent attachment of immunoglobulin fragments on liposomal integrity. *Biochemistry.* 1986;25:5693–5698.

26. Spragg DD, Alford DR, Greferath R, et al. Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system. *Proc Natl Acad Sci USA.* 1997;94:8795–8800.

27. Cheresh DA. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc Natl Acad Sci USA.* 1987;84:6471–6475.

28. Czyz M, Cierniewski CS. Selective Sp1 and Sp3 binding is crucial for activity of the integrin alpha v promoter in cultured endothelial cells. *Eur J Biochem.* 1999;265:638–644.

29. Koning GA, Morselt HW, Velinova MJ, et al. Selective transfer of a lipophilic prodrug of 5-fluorodeoxyuridine from immunoliposomes to colon cancer cells. *Biochim Biophys Acta.* 1999;1420:153–167.

30. Goren D, Horowitz AT, Zalipsky S, Woodle MC, Yarden Y, Gabizon A. Targeting of stealth liposomes to erbB-2 (Her/2) receptor: in vitro and in vivo studies. *Br J Cancer.* 1996;74:1749–1756.

31. Kirpotin D, Park JW, Hong K, et al. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry.* 1997;36:66–75.

32. Yuan F, Dellian M, Fukumura D, et al. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.* 1995;55:3752–3756.