Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1768 (2007) 427–438
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Development and characterization of magnetic cationic liposomes for targeting tumor microvasculature

Suman Dandamudi, Robert B. Campbell *

Northeastern University, 360 Huntington Avenue, Bouvé College of Health Sciences, Department of Pharmaceutical Sciences, 110 Mugar Hall, Boston, MA 02115, USA

Received 5 July 2006; received in revised form 11 October 2006; accepted 12 October 2006
Available online 21 October 2006

Abstract

Cationic liposomes preferentially target tumor vasculature compared to vessels in normal tissues. The distribution of cationic liposomes along vascular networks is, however, patchy and heterogeneous. To target vessels more uniformly we combined the electrostatic properties of cationic liposomes with the strength of an external magnet. We report part I of development. We evaluated bilayer physical properties of our preparations. We investigated interaction of liposomes with target cells including the role of PEG (polyethylene-glycol), and determined whether magnetic cationic liposomes can respond to an external magnetic field. The inclusion of relatively high concentration of MAG-C (magnetite) at 2.5 mg/ml significantly increased the size of cationic liposomes from 105±26.64 to 267±27.43 nm and reduced the zeta potential from 64.55±16.68 to 39.82±5.26 mv. The phase transition temperature of cationic liposomes (49.97±1.34 °C) reduced with inclusion of MAG-C (46.05±0.21 °C). MAG-C cationic liposomes were internalized by melanoma (B16-F10 and HTB-72) and dermal endothelial (HMVEC-d) cells. PEG partially shielded cationic charge potential of MAG-C cationic liposomes, reduced their ability to interact with target cells in vitro, and uptake by major RES organs. Finally, application of external magnet enhanced tumor retention of magnetic cationic liposomes.

Keywords: Cancer; Melanoma; Tumor vasculature; Magnetic drug targeting; Magnetic cationic liposome; Physical characterization

1. Introduction

More than 23% of all human deaths in the United States are associated with cancer [1]. The development of effective treatment strategies is therefore of vital importance, and is urgently needed. A popular clinical approach targets functional tumor vessels in an effort to destroy the existing tumor vasculature. A common goal of all successful vascular targeting strategies is the ability to interrupt the flow of oxygen and nutrients to the developing tumor mass. Tumor vessels are the main focus of this therapeutic approach, and cancer cells die as a result of vascular injury [2].

One potential problem associated with the use of vascular targeting strategies is the lack of specificity. For this reason, the ability to reduce the total amount of drug delivered to healthy tissues, while improving selective delivery to tumor targets is a formidable challenge. Success with chemotherapy is thus dependent on the correct identification of therapeutic agents, as well as accurate identification and appropriate use of strategies to deliver them to targets [3].

Another popular approach is magnetic drug targeting (MDT) [4–6]. MDT improves selective delivery of chemotherapeutic agents to tumors with the use of ferro fluids bound to drugs that

Abbreviations: DMPC, 1,2-Dimyristoyl-phosphatidylcholine; DMTAP, 1,2-Dimyristoyl-trimethyl-ammonium propane; DMPE-PEG5000, 1,2-Dimyristoyl-phosphatidylethanolamine-polyethylene glycol; CHOL, Cholesterol; MAG-C, Fluid MAG-CS (magnetite, citric acid matrix); DPH, 1,6-diphenyl-1,3,5-hexatriene; DMEM, Dulbecco's Modified Eagle Medium; MEME, Eagles Minimum Essential Medium; EBM-2, Endothelial Cell Basal Medium-2; HMVEC-d, Human Dermal Microvascular Endothelial Cells; HMEC-1, Human Dermal Microvascular Endothelial Cells; HUVEC, Human Umbilical Vein Endothelial Cells; B16-F10, Murine melanoma; HTB-72, Human melanoma; SRB, Sulforhodamine B

* Corresponding author. Northeastern University, 360 Huntington Avenue, Bouvé College of Health Sciences, Department of Pharmaceutical Sciences, 31 Mugar Hall, Boston, MA 02115, USA. Tel.: +1 617 373 3091; fax: +1 617 373 8886.
E-mail address: r.campbell@neu.edu (R.B. Campbell).

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.10.006

can respond to an external magnetic field [6,7]. In the MDT strategy, the magnetic field retains the chemotherapeutic agent at the intended site of drug action, and therefore increases drug levels at this location. This minimizes the potential for accumulation of drug in healthy tissues. Furthermore, the combination of improved target selectivity, and enhanced duration of drug exposure to target consequently reduce the overall amount of drug taken up by the RES (reticuloendothelial system) [5].

A prerequisite for any successful magnetic drug targeting approach is that the ferro fluid–drug complex must reach the tumor microcirculation and release the drug at this location [8,9]. The most important parameters to be considered for magnetic drug targeting are [5,10]: (1) the concentration, and type of ferro fluid employed, (2) the magnetic strength of the external magnetic field, (3) and the length of time the target tissue is exposed to the external magnet. All three parameters should be carefully selected and optimized for each purpose.

A more recent contribution to the field of targeted drug delivery involves the use of cationic liposomes [11–14]. Cationic liposomes, unlike their anionic and electroneutral counterparts, have been shown to target tumor vessels to a significant extent over vessels in normal healthy tissues, targeting approximately 25 and 5% of vessel areas respectively [14]. Although cationic liposomes preferentially accumulate in tumors, the distribution of liposomes along tumor vessels is non-uniform. Therefore, preferential sites of drug accumulation are observed, and although many vessels are targeted some vessel areas are not targeted by this approach.

We seek to develop one delivery vehicle that is (1) relatively tumor specific, (2) capable of interacting with tumor vessels, (3) and capable of responding to an external magnetic field. Ultimately we hope to improve tumor vascular interactions with cationic liposomes by forcing them to interact uniformly with the tumor vasculature. By this approach we seek to overcome the heterogeneity associated with the use of cationic liposomes alone.

We report the first step of the development and characterization of MAG-C (magnetite) cationic liposomes. We specifically investigated the effect of MAG-C on bilayer physical properties of cationic liposomes. We determined the extent to which they associate with, and are internalized by, murine tumor (B16-F10), human tumor (HTB-72), and Human Microvascular Dermal Endothelial (HMVEC-d) cells in the presence and absence of an external magnet. Lastly, we discuss the implications of our in vitro and in vivo studies in terms of tumor vascular targeting.

2. Experimental procedures

2.1. Materials

Dimyristoyl-phosphatidylcholine (DMPC), Dimyristoyl-trimethyl ammonium propane (DMTAP), cholesterol, rhodamine-DPPE (N-Lissamine rhodamine B sulfonyl)-dipalmitoyl-phosphatidylethanolamine) (Ammonium salt), DMPE-PEG 5000 purchased from Avanti Polar Lipids (Alabaster, AL); fluid MAG-C (aqueous dispersion of magnetite (Fe_3O_4) from Chemicell (Berlin, Germany); DPH (1, 6 diphenyl-1, 3, 5 hexatriene) purchased from Molecular Probes (Eugene, OR, USA), permanent magnets were purchased from Master Magnetics, Inc (Castle Rock, Colarado). For in vitro and in vivo experiments, ceramic (0.4 T (Tesla) and neodymium (1.2 T) magnets were used, respectively.

2.2. Cell culture and media

B16-F10 (CRL 6475) and SK-MEL-28 (HTB-72) cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA). B16-F10 was maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS). SK-MEL-28 was cultured in MEME with 10% FBS. HMVEC-d (human dermal microvascular endothelial cells) was maintained in EBM-2 with additional required growth supplements both purchased from Cambrex Bio Science (Walkersville, MD). All cell line cultures were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Preparation of liposomes

The components used to prepare liposomes were determined by the experimental requirements. DMPC, DMTAP, cholesterol, and rhodamine-DPPE represent the standard components used to prepare liposomes unless otherwise stated. The required amount of lipid (typically around 10 μmol/ml) was dissolved in chloroform, and then transferred to a pyrex tube (or round bottom flask). The solvent in the tube was subsequently removed using a rotary evaporator. The temperature was held slightly above the phase transition temperature of the dominant lipid in mixture until a thin film was deposited on the inside wall of the tube. The lipid film was then allowed to freeze-dry for 4 h. The dried lipid film was hydrated with saline, different concentrations of MAG-C (0.5 mg/ml, 2.5 mg/ml) in saline. The specific volume added to the dry film depended on experimental purpose. The newly formed preparation was warmed to a temperature slightly above the phase transition temperature of the dominant lipid of preparation, and vortexed intermittently until the lipid film was completely resuspended [15]. The composition of various liposomal formulations is shown in Table 1. To produce a homogenous mixture of small unilamellar vesicles, liposome preparations were sonicated in a bath type sonicator (Laboratory Supplies Corporation, Hicksville, NY). Unincorporated MAG-C was separated from the MAG-C associated variety by centrifugation at 1000×*g* for 15 min [16,17]. Determination of liposome size and zeta potential was achieved with use of a 90 Plus Particle/ Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY).

2.4. Fluorescence polarization

The phase transition temperature (indicator of membrane fluidity) of liposomes was calculated by the DPH fluorescence polarization method discussed previously [18,19]. Briefly, DPH was dissolved in THF to give a concentration of 1 mg/ml. The stock solution of liposomes (10 μmol/ ml) was diluted to give a final concentration of 1 μmol/ml. Approximately 2 ml of diluted liposome stock was mixed with 10 μl of DPH (1 mg/ml). The sample was wrapped in aluminum foil and incubated at 37 °C for 30 min in an ISOTEMP 215 type water bath from Fisher Scientific (Pittsburgh, PA), and rigorously vortexed in the absence of direct light. Fluorescence measurements were performed using a scanning spectrofluorometer from Photon Technology International (PTI) with a Peltier rapid temperature change element built into

Table 1
Preparation of liposomal formulations

Group	Liposome preparation	Lipid ratio (mol%)
1	DMPC	100
2	DMPC/DMTAP	50:50
3	DMPC/DMTAP/CHOL	40:50:10
4	DMPC/DMTAP/CHOL/PEG	35:50:10:5
5	DMPC/DMTAP/CHOL/MAG-C	40:50:10
6	DMPC/DMTAP/CHOL/MAG-C/PEG	35:50:10:5

cuvette holder (Lawrenceville, NJ). Measurements were made at an excitation wavelength of 355 nm, emission wavelength of 430 nm, 4 nm of excitation and emission slits, and FP110 film polarizers. Fluorescence polarization was calculated using the following equation [19]:

$$P = [\{I(0,0) - G\} \times I(0,90)] / [\{I(0,0) + G\} \times I(0,90)\}],$$
$$G = I(90,0) / I(90,90)$$
$$\text{Percent gel phase} = P/0.4 \times 100$$

2.5. Cytotoxicity studies

The percent of cell viability was determined by sulforhodamine B assay [20]. Cells were seeded at 1×10^4 cells/ml in each well of a 48 well plate (Fisher Scientific, Pittsburgh, PA). Cells were allowed a sufficient amount of time to adhere to well plate and cells were treated with different amounts of liposomes (typically ranging from 10 to 1000 nmol). After 24 h, well plates containing cells were washed with PBS to remove unbound liposomes and cellular debris. Cells were fixed to plate by adding 100 μl of 50% TCA into each well followed by storage at 4 °C for a 1 h period. After 1 h each well was washed 4 times with distilled water and then cells were stained with 200 μl of 0.4% sulforhodamine B for 30 min. To remove unbound stain, wells were washed with 1% acetic acid and air-dried in a laminar flow hood [20,21]. Fluorescence intensity (FI) was measured by dissolving bound sulforhodamine with 1 ml of 1× PBS at an excitation wavelength of 550 nm, and emission wavelength of 590 nm using a FLX 800 Fluorescence Microplate Reader, Bio-Tek Instruments Inc., (Winooski, Vermont). Percent of cell viability was calculated by the following formula:

$$\text{Percent of cell viability} = \frac{\text{F.I. of treated cell population}}{\text{F.I. of untreated cell population}} \times 100$$

2.6. Cell–liposome interactions: quantitative analysis

To measure the extent of liposomes associated with cells, all cells were seeded at 1×10^4 cells/ml in a 48 well plate. Cells were allowed to adhere to the plate overnight and then various amounts of rhodamine labeled liposomes (10–1000 nmol) were added. After 24 h the cells were washed with PBS to remove unbound liposomes. The total fluorescence was measured by a FLX 800 Fluorescence Microplate Reader. Analysis of total rhodamine labeled liposome uptake was determined as discussed in Cytotoxicity studies.

2.7. In vitro interaction of MAG-C liposomes in presence of external magnet

To determine the influence of external magnet on the extent of association of magnetic liposomes with murine melanoma and endothelial cells, cells were seeded at 4×10^4 cells/ml in a 12 well plate. Cells were allowed to adhere to the plate overnight and then rhodamine labeled magnetic cationic and electroneutral liposomes (500 nmol) containing two different concentrations of MAG-C (0.5 mg/ml and 2.5 mg/ml) were added to each well. Electroneutral liposomes consisted of DMPC and cholesterol (50:50) and method of preparation is described in Preparation of liposomes. A permanent magnet of strength 0.4 T was placed under the cell culture plate for 1 h. For this period of 1 h the well plate was placed on a Stovall Belly Dancer Shaker (Fisher Scientific, NJ) that was set on slow and continuous rotation. After 1 h the plate was removed from magnet and shaker. Following an additional 23 h incubator cells were immediately washed with PBS to remove unbound liposomes. The total fluorescence was measured by a FLX 800 Fluorescence Microplate Reader. The percent association of magnetic liposomes with cells was calculated as discussed in Cytotoxicity studies.

2.8. Fluorescence microscopy

Fluorescence microscopic analyses were carried out using a BX61 WI Olympus fluorescence microscope from Optical Analysis Corporation (Melville, NY). Liposomes labeled with rhodamine-DPPE (N-Lissamine rhodamine B sulfonyl)-dipalmitoyl-phosphatidylethanolamine) were used to investigate the nature of liposome–cell interactions. Cells were seeded on sterile cover slips in 6 well plates at a density of 5×10^5 cells/ml. The cells were allowed to adhere on the cover slips for 24 h in required growth medium. Cells were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Cells were then exposed to 100 nmol of rhodamine labeled liposomes for 24 h following the initial incubation period. Twenty-four hours later the required growth media was removed, cells were washed with PBS to separate unbound liposomes from the cellular-bound fraction. DIC (Differential Interference Contrast) microscopy was used to observe cell shape and morphology prior to obtaining all fluorescence images. Rhodamine channel was used to identify labeled liposomes in cells under 20× and 40× magnifications. DIC and fluorescence images were finally merged or blended to observe association and/ or internalization of liposomes with respect to the cell.

2.9. Biodistribution studies

Tissue distribution studies were performed in male SCID mice. B16-F10 cells were injected subcutaneously into male SCID mice between 8 and 10 weeks old. An average tumor volume of approximately 250 mm^3 was achieved 10 days post injection of cells. Approximately 0.1 ml of ^{111}In labeled MAG-C and PEGylated MAG-C cationic liposomes (20 μmol/ml) was administered systemically via tail vein injection. Approximately 24 h post injection the mice were anesthetized with isoflurane. Blood was collected from the retro-orbital sinus. Mice were then sacrificed by cervical dislocation and liver, lung, spleen and tumor were excised. The weight of each organ was measured and the amount of radioactivity recovered by each organ was determined with the use of Beckman Gamma 5500 B counter (Fullerton, CA). Results were expressed as percent of injected dose per gram of tissue. Percent of radioactivity in each organ was calculated as follows.

$$= \frac{\text{CPM values in each organ/weight in gm}}{\text{CPM values of injected dose}} \times 100$$

2.10. In vivo tumor accumulation of MAG-C cationic liposomes in presence of external magnet

B16-F10 cells were injected subcutaneously into SCID mice approximately 8–10 weeks old, and tumor was allowed to grow until tumor volume reached 250 mm^3. B16-F10-bearing mice were divided into 4 groups. Group 1 and 3 received intravenous administration of MAG-C cationic liposomes without application of an external magnet. Group 2 and 4 received injection of preparation but tumor had access to externally applied (neodymium) magnet for a period of 1 h. Mice in groups 1 and 2 were sacrificed immediately following removal of magnet. Mice in group 3 and 4 were sacrificed and organs removed 1 h following the removal of magnet (or 2 h post-systemic injection of MAG-C cationic liposomes). Tumor weight was measured and the amount of radioactivity recovered was determined with a Beckman Gamma 5500 B counter. Results were expressed as the percent of injected dose per gram of tissue. Percent of radioactivity in each organ was calculated as per the equation described above.

2.11. Statistical analysis

The non parametric Mann–Whitney U test was used to evaluate statistically significant differences in the experimental groups. P values <0.05 were considered statistically significant.

3. Results

3.1. Physicochemical characterization

Liposome size, charge, and membrane fluidity have previously been shown to influence drug retention, intratumoral distribution, and factors associated with the rate of elimination

of liposomes from circulation [13–15,18,19,22–25]. For this reason we investigated liposome size, zeta potential (surface charge potential), and phase transition temperature of potentially useful preparations. Several ingredients were used to prepare MAG-C cationic liposomes. The extent to which each component exerted its effect on bilayer physical properties was determined (see Tables 1 and 2).

3.2. Liposome size

Changes in liposome size and dispersity are important indicators of change in bilayer properties of liposomes. The following values reported for liposome size were determined following 10 min of sonication. The size of DMPC liposomes (483±187.02 nm) tended toward smaller mean diameters when the cationic lipid DMTAP was included as a component of the preparation. However, this decrease was not statistically significant ($P>0.05$). We also investigated the effect of including cholesterol in DMPC/DMTAP preparations; cholesterol significantly reduced liposome diameter of DMPC/DMTAP liposomes from 369±144.38 nm to 105±26.64 nm. When MAG-C (magnetite) was added at 0.5 mg/ml there was no significant change in mean diameter of DMPC/DMTAP/cholesterol liposomes, but higher MAG-C content (2.5 mg/ml) resulted in a significant increase in mean diameter from 105±26.64 nm to 267±27.43 nm.

3.3. Zeta potential

Quantitative changes in surface charge characteristics of liposomes due to the inclusion of different liposome components can be used to predict the relative affinity of tumor vessels for liposomes on the basis of charge. Zeta potential values were determined immediately following size measurements, and were therefore the identical preparations used for the determination of size. Zeta potential for electroneutral liposomes consisting of DMPC alone was −0.26±3.99 mv. This value increased significantly in the presence of 50 mol% DMTAP (84.96±15.57 mv; $P<0.05$). The inclusion of 10 mol% cholesterol in DMPC/DMTAP preparations reduced zeta potential from 84.96±15.57 to 64.55±16.68 ($P>0.05$). The inclusion of 0.5 mg/ml of MAG-C into cationic liposomes significantly reduced the zeta potential of MAG-C cationic liposomes (43.79±10.93 mv) compared to preparations without MAG-C (64.55±16.68 mv). An increase in MAG-C (2.5 mg/ml) content resulted in no significant changes in zeta potential compared to preparations containing 0.5 mg/ml of MAG-C.

3.4. Membrane fluidity

DPH (diphenylhexatriene) can be used to determine specific changes in membrane fluidity due to components added to the liposome preparation. The fluorescent membrane probe is located deep within the core of the liposome bilayer, where it can monitor changes in membrane phase behavior [26]. Due to its specific location sudden changes in its rotational freedom can be used to calculate the fraction of gel and liquid crystalline phase at any given temperature [18,19,26]. We therefore used DPH to investigate the fluidizing effect of MAG-C and various lipids exerted on bilayer phase state.

Components used to investigate effects on size and zeta potential were used here to investigate effects on membrane fluidity. As with effects on size and zeta potential, each liposome type represents the inclusion of one additional component. For this reason, additional changes observed in the rotational freedom of DPH in the bilayer will be used to determine how each component alters bilayer phase state.

Fluorescence polarization values of approximately 0.45 and 0.0 correspond to 100% of gel and liquid crystalline phase of the liposome bilayer, respectively. In liposomes of DMPC alone, DPH had a polarization value of approximately 0.31 at 12 °C and a phase transition temperature of 26 °C. The inclusion of DMTAP (50 mol%) significantly increased the polarization values to 0.45 at 12 °C. The phase transition temperature for liposomes of DMPC/DMTAP was 42 °C. DMTAP therefore reduced the fraction of liquid crystalline phase compared to DMPC alone, as observed between temperatures of 12 to 45 °C. After 45 °C DMPC and DMPC/DMTAP liposomes had similar polarization values and existed predominately in the liquid crystalline phase state (Fig. 1A). Incorporation of cholesterol (10 mol%) increased the phase transition temperature of DMPC/DMTAP liposomes from 42 °C to 50 °C. The bilayer properties became more rigid in the presence of cholesterol compared to liposomes of DMPC/DMTAP alone (Fig. 1B). Our data support this conclusion showing that higher temperatures were required to melt the lipid bilayer in the presence of cholesterol. The phase

Table 2
Physical characterization of liposomes: the size and zeta potential values for different liposomal preparations were determined immediately following 10 min of sonication

Group	Liposome preparation	Size (nm)	Zeta potential (mv)	Group comparisons	P values	
					Size	Zeta potential
1	DMPC	483±187.02	−0.26±3.99	–	–	–
2	DMPC/DMTAP	369±144.38	84.96±15.57	1 and 2	NS	$P<0.05$
3	DMPC/DMTAP/CHOL	105±26.64	64.55±16.68	2 and 3	$P<0.01$	NS
4	DMPC/DMTAP/CHOL/PEG	109±5.31	26.24±2.28	3 and 4	NS	$P<0.01$
5	DMPC/DMTAP/CHOL/MAG-C (0.5 mg/ml)	142±27.40	43.79±10.93	3 and 5	NS	$P=0.05$
6	DMPC/DMTAP/CHOL/MAG-C/PEG	141±14.52	21.98±5.25	5 and 6	NS	$P<0.01$
7	DMPC/DMTAP/CHOL/MAG-C (2.5 mg/ml)	267±27.43	39.82±5.26	3 and 7; 5 and 7	$P<0.05$; $P<0.05$	$P<0.05$ NS
8	DMPC/DMTAP/CHOL/MAG-C/PEG	156±16.25	27.56±3.54	7 and 8	$P<0.05$	$P<0.05$

MAG-C containing liposomes were centrifuged at 1000×g for 15 min to remove unincorporated MAG-C.

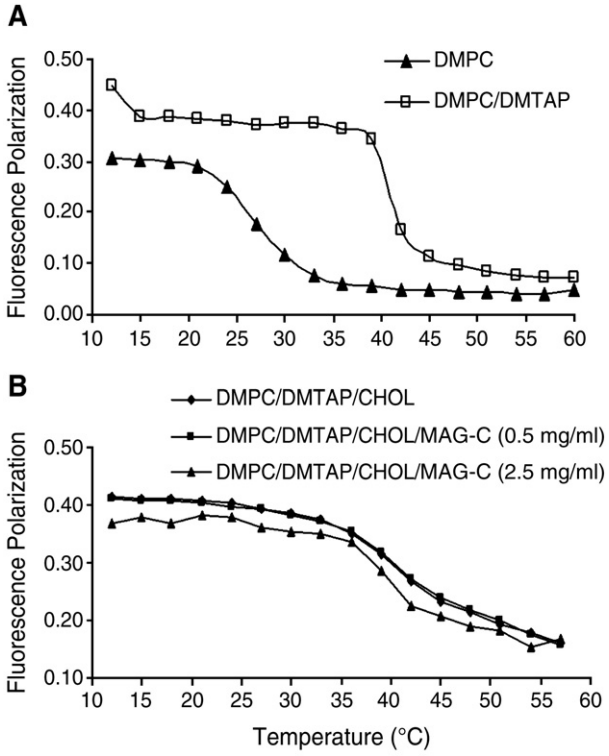


Fig. 1. Fluorescence polarization (FP) as a function of temperature for magnetic liposome preparations: DPH was incorporated into bilayers of different liposomal formulations and fluorescence intensity values were recorded at various temperatures at a fixed excitation and emission wavelength of 355 nm and 430 nm respectively. FP values were plotted as a function of temperature for each liposomal formulation and the values for FP were determined as discussed in Experimetal procedures.

transition temperature of cationic liposomes was not altered by the inclusion of low concentrations of MAG-C (0.5 mg/ml) (Fig. 2). As the MAG-C concentration was increased to 2.5 mg/ml the phase transition temperature decreased from 50 °C to 46 °C.

3.5. Cell viability assay

We evaluated the effect of MAG-C cationic liposomes on cell viability. We observed no toxic effects on cell growth against murine melanoma (B16-F10), human melanoma (HTB-72), and human dermal microvascular endothelial (HMVEC-d) cells at

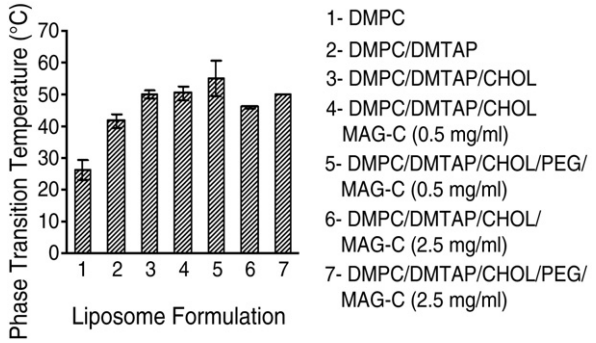


Fig. 2. Phase transition temperature of different liposomal formulations. Based on the polarization values, percent gel phase was calculated as described in Experimetal procedures.

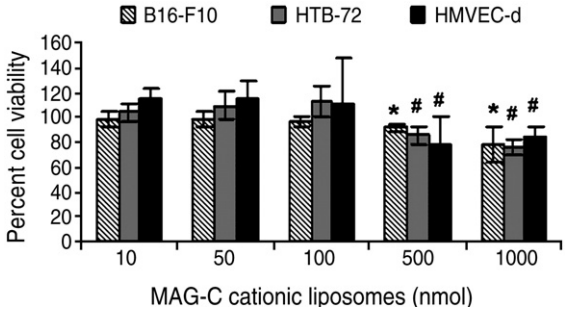


Fig. 3. Percent cell viability of melanoma and endothelial cells treated with MAG-C cationic liposomes: Cells were seeded at 1×10^4 cells/ml in a 48 well plate and incubated at 37 °C. Percent of cell viability was determined following 24 h of exposure to various amounts of magnetic cationic liposomes (10–1000 nmol). Percent cell viability was calculated for each liposome concentration based on the formula provided in Experimetal procedures. The control group was untreated. Each value represents the mean ± S.D. of 7 different experimental determinations (*$P<0.05$ and $^{\#}P<0.01$ compared to 10 nmol).

<1000 nmol (Fig. 3). The percent of viable B16-F10, HTB-72 and HMVEC-d cells after 24 h exposure to 500 nmol of liposomes was 92, 85, and 78% respectively (Fig. 3). Although the values reported here for HMVEC-d tended toward relatively lower cell viabilities, no statistically significant effects were observed. The percent of viable cells remaining in culture after exposure to MAG-C cationic liposomes reduced significantly at ≥500 nmol compared to 10 nmol with all three cell lines. Moreover, we observed no significant difference in cell viability among cell lines at any given concentration, suggesting that the liposomes were equally toxic to both cancer and microvascular endothelial cells.

3.6. Cell association studies

The tendency of MAG-C cationic liposomes to associate with cells increased with liposome amount, and association values were similar for the three cell lines. We observed no significant increase in cell association >500 nmol of liposomes (Fig. 4).

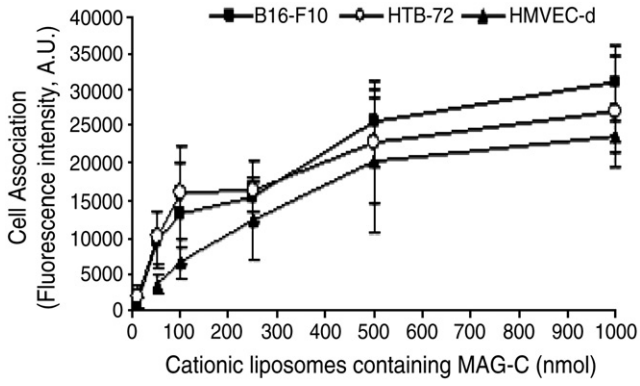


Fig. 4. Association of MAG-C cationic liposomes with melanoma and endothelial cells *in vitro*: Cells were seeded at 1×10^4 cells/ml in a 48 well plate and incubated at 37 °C. The relative association of cells with each liposome preparation type was determined 24 h following cell exposure to rhodamine labeled liposomes (10–1000 nmol). The control group was untreated. Each value represents the mean ± S.D. of 6 different determinations.

To observe direct cellular uptake of MAG-C cationic liposomes we used DIC and fluorescence microscopic methodology. Images were acquired with the use of each technique, and were merged to assess site(s) of accumulation relative to cellular organelles (Fig. 5B). Avid accumulation of MAG-C cationic liposomes was evident in all cell lines with the most significant uptake observed in B16-F10 and HTB-72. In addition we note perinuclear uptake in HMVEC-d cells.

3.7. Role of PEG in MAG-C cationic liposomes

A sterically stabilized liposome is one that is surface coated with polyethylene glycol (PEG), or some other polymer [27]. The purpose of including PEG in preparations is to limit the interaction of liposomes with opsonins in blood; acquisition to proteins in blood rapidly eliminates them from systemic circulation [25]. Liposomes bearing a high cationic surface charge potential are the most sensitive to this mechanism of elimination, and so our MAG-C cationic liposomes could suffer a similar fate. Prolonging the circulation half-life of MAG-C cationic liposomes should increase overall tumor vascular targeting efficiency due to extended access to the external magnetic field. We therefore investigated the effect of PEG on bilayer physical properties.

We determined the effect of 5 mol% DMPE-PEG on size, and zeta potential, of cationic liposomes containing MAG-C. The following studies compared MAG-C cationic liposomes to MAG-C PEGylated cationic liposomes (MAG-C PCLs). The

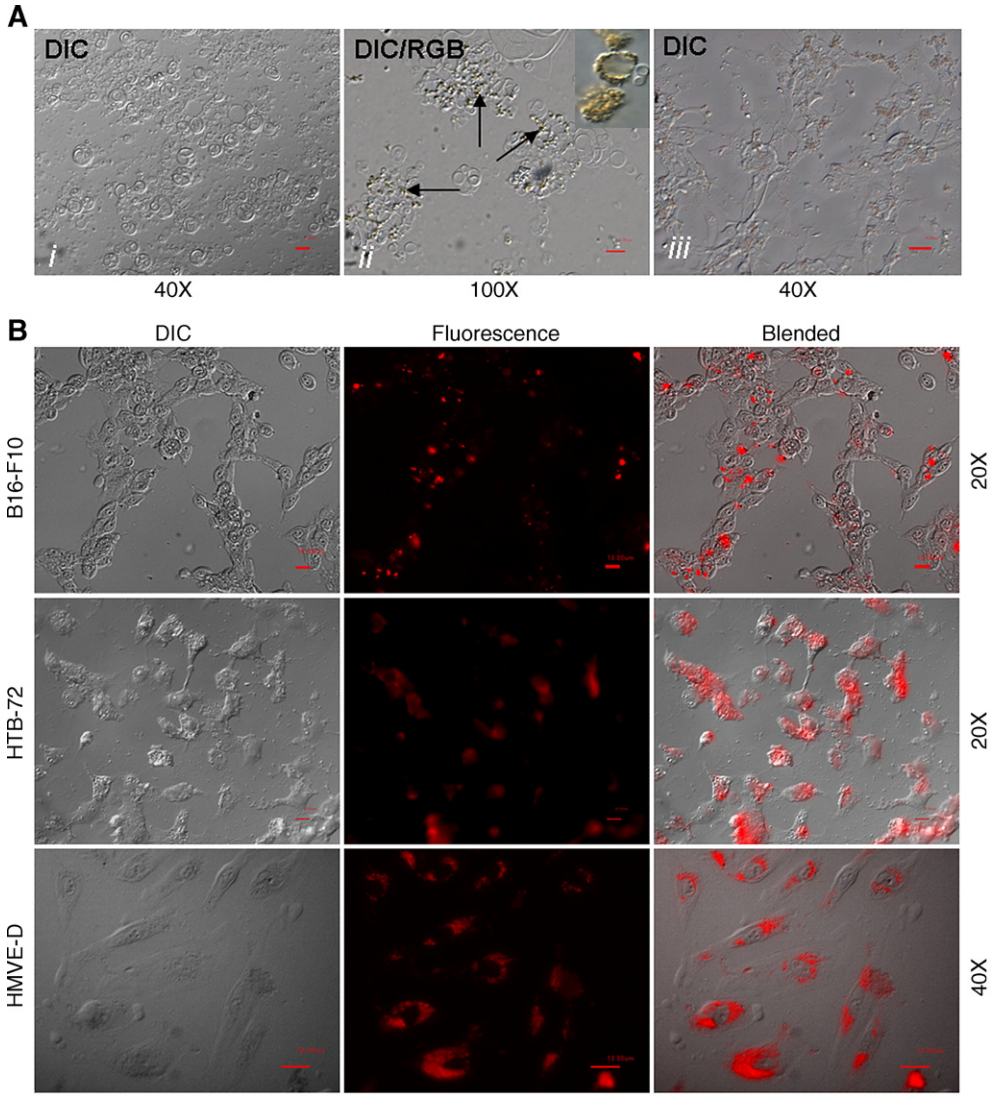


Fig. 5. Analysis of MAG-C association with liposomes: Liposomes were prepared as discussed in Experimetal procedures. Images were acquired by DIC microscopy and RGB. (A) Incorporation of MAG-C in cationic liposomes: (i) cationic liposomes (DMPC/DMTAP/cholesterol) under DIC (40×), (ii) DIC image of MAG-C cationic liposomes under RGB filter (100×), (iii) DIC image of MAG-C cationic liposomes taken up by B16-F10 cells under RGB filter (40×). Arrows indicate MAG-C. (B) Intracellular uptake of MAG-C cationic liposomes: Cells were seeded at 5×10^5 cells/ml in a 6 well plate. Cells were treated with rhodamine labeled MAG-C cationic liposomes for 24 h at 37 °C with 100 nmol of liposomes. Rhodamine labeled MAG-C cationic liposomes are indicated in red. The blended image of fluorescence and DIC show the localization of MAG-C in cells. Magnification setting for B16-F10 and HTB-72 was 20×, and for HMVEC-d it was 40×.

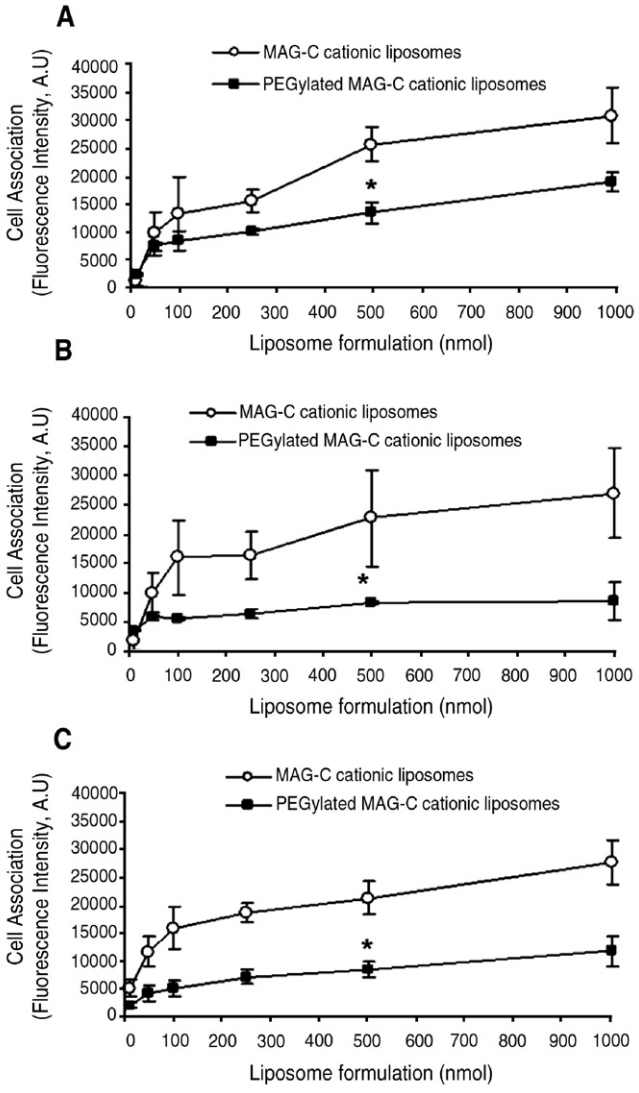


Fig. 6. Cell association—comparison of MAG-C cationic liposomes versus PEGylated cationic liposomes (PCLs) containing MAG-C: cells were seeded at 1×10^4 cells/ml in a 48 well plate and incubated at 37 °C. Association measurements were determined 24 h following exposure of cells to various amounts of rhodamine labeled MAG-C cationic liposomes, and MAG-C PEGylated cationic liposomes (10–1000 nmol). The control group was untreated. MAG-C PEGylated cationic liposomes interacted with all three cell lines to a significantly less extent compared to MAG-C cationic liposomes without the inclusion of PEG. Each value represents the mean±S.D. of 3 sets of determination, $P<0.01$. (A) B16-F10 cells, (B) HTB-72, (C) HUVEC.

size of MAG-C PCLs (containing 0.5 mg/ml) was 142.56±14.22 and was similar to values reported for MAG-C liposomes in absence of PEG (141.143±31.55). PEG reduced zeta potential of cationic liposomes containing MAG-C, showing a value of 21.98±5.25 mv compared to preparations without PEG (43.79±10.93 mv). The inclusion of DMPE-PEG to the cationic liposome preparation containing relatively high MAG-C (2.5 mg/ml) content resulted in a significant decrease in liposome size from 262.67±27.43 to 156±16.25 nm.

We next compared the interaction of PCLs with cancer (B16-F10 and HTB-72) and endothelial (HUVEC) cell lines. We observed that the inclusion of PEG significantly reduced cellular interactions regardless of cell type (Fig. 6).

These data suggest that the inclusion of 5 mol% PEG altered bilayer physical properties such as liposome cationic surface charge potential, but did not affect liposome size. Finally, PEG limited the association of MAG-C cationic liposomes with all cell lines; reduced interactions may be due to the reported decrease in zeta potential ($P<0.01$).

3.8. In vivo tissue distribution profile of ^{111}In labeled MAG-C cationic and PEGylated MAG-C cationic liposomes

We next determined the effect of PEG on tissue distribution profile of MAG-C cationic liposomes in melanoma bearing SCID mice. When MAG-C cationic liposomes were injected intravenously into melanoma bearing mice, the percent of injected dose per gram of the tissue in lung, liver and spleen was 35.27, 56.61 and 75.92% respectively (Fig. 7). The inclusion of 5 mol% DMPE-PEG resulted in a significant reduction in the uptake by the major organs of RES. The significant reduction was due to the electrostatic repulsive effect of PEG [25,27]. Moreover, tumor/blood ratio for PEGylated MAG-C cationic liposomes (4.36±1.14) was significantly higher than that for MAG-C cationic liposomes (1.56±0.43). The data suggest that PEG minimizes uptake of MAG-C cationic liposomes by healthy tissues while improving tumor uptake.

3.9. Cellular interactions of MAG-C liposomes in presence of magnet

We have noted that MAG-C can be successfully incorporated in cationic liposomes however, whether or not the concentration is sufficiently available in liposomes to respond to an external magnet has yet to be established. For this reason, cellular interaction of MAG-C liposomes was studied in the presence and absence of an external magnet in vitro. From the cell association studies of MAG-C liposomes with B16-F10 and HMEC-1 cells we observed that there was no significant

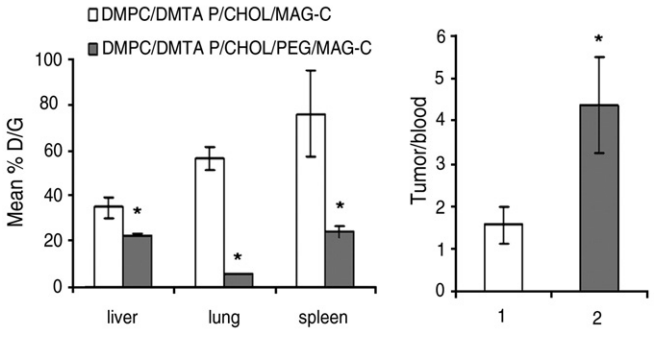
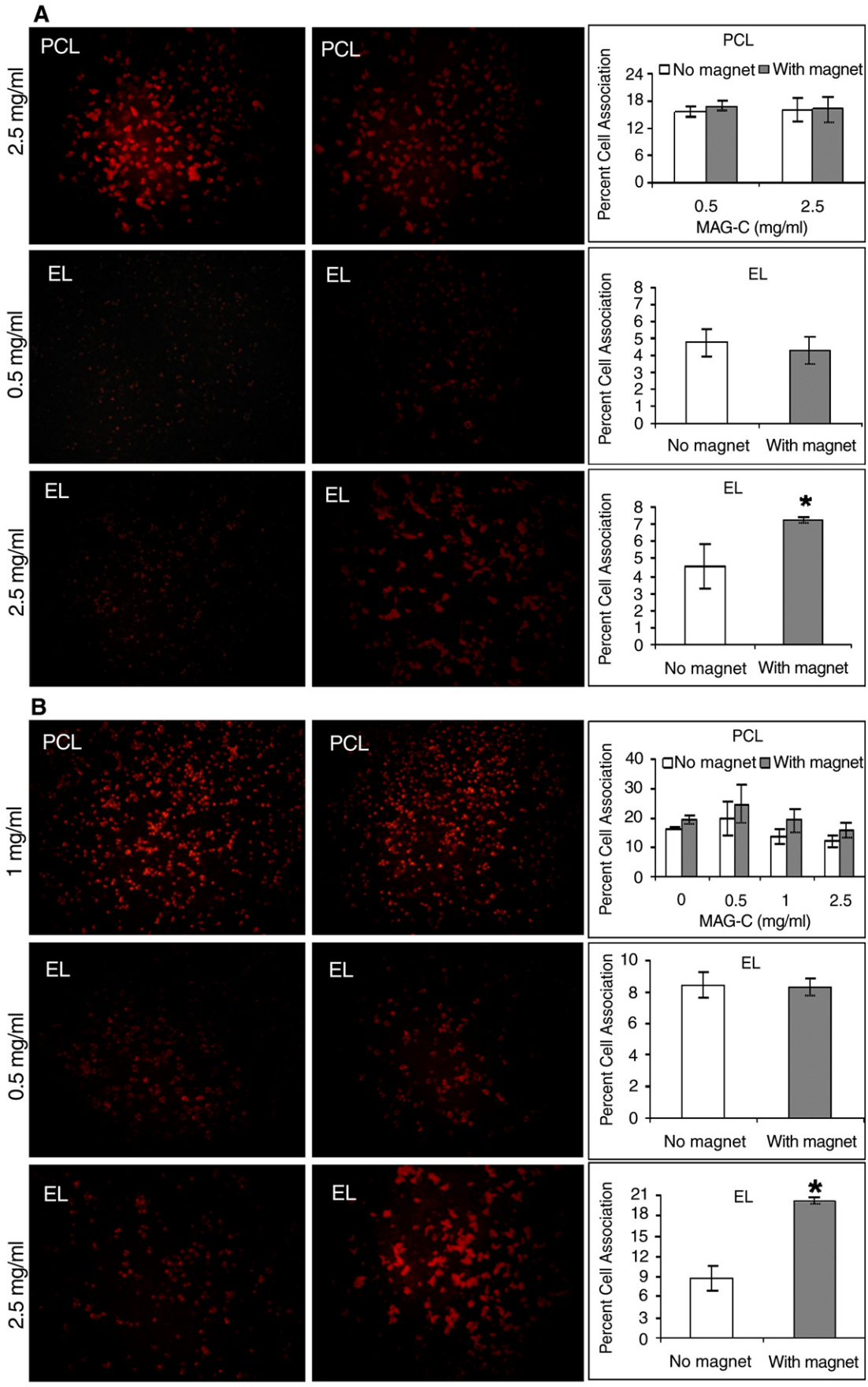


Fig. 7. In vivo tissue distribution profile of ^{111}In labeled MAG-C cationic and PEGylated MAG-C cationic liposomes in mice bearing subcutaneous melanoma tumors of approximately 250 mm^3 volume. Mice were injected with ^{111}In labeled liposomes. 1—MAG-C cationic liposomes, 2—PEGylated MAG-C cationic liposomes. Twenty-four hours post injection of each preparation type, percent of label recovered by liver, lung, spleen, blood and tumor was determined. Each value represents mean±S.D. of 4 animals per group, $P<0.05$.


A
2.5 mg/ml
PCL
PCL
PCL
No magnet
With magnet
Percent Cell Association
24
18
12
6
0
0.5
2.5
MAG-C (mg/ml)
0.5 mg/ml
EL
EL
EL
8
7
6
5
4
3
2
1
0
No magnet
With magnet
2.5 mg/ml
EL
EL
EL
*
No magnet
With magnet
B
1 mg/ml
PCL
PCL
PCL
No magnet
With magnet
40
30
20
10
0
0
0.5
1
2.5
MAG-C (mg/ml)
0.5 mg/ml
EL
EL
EL
10
8
6
4
2
0
No magnet
With magnet
2.5 mg/ml
EL
EL
EL
*
21
18
15
12
9
6
3
0
No magnet
With magnet

increase in the interaction of liposomes above 500 nmol, and that the minimal amount of toxicity associated with the use of this amount was acceptable. We therefore compared the cell association of MAG-C liposomes (500 nmol) in presence and absence of an external magnet (0.4 T).

The association of MAG-C PCLs (containing 0.5 mg/ml of MAG-C) with B16-F10 cells without the external magnet was 16±1.19% and was similar to interaction in presence of magnet (17±1.17%) (Fig. 8). We also noted no significant benefit of using the external magnet at 2.5 mg/ml of MAG-C.

The ability of cationic liposomes to be internalized by cells in absence of magnet may have overshadowed the benefit of using the external magnet under the current experimental setup. We therefore evaluated the ability of MAG-C electroneutral liposomes (containing 0.5 mg/ml of MAG-C) to associated with B16-F10 cells in presence of the external magnet. The result was 4.78±0.8% which was similar to when cells were exposed to electroneutral liposomes without the magnet. We however observed an approximate 2 fold significant increase in association of MAG-C electroneutral liposomes from 4.55±1.28% to 7.25±0.19% with the external magnet at 2.5 mg/ml MAG-C.

We next carried out the same study using human dermal endothelial cells, our in vitro model of capillary networks. The interaction of MAG-C PCLs (20±6%) to the vascular cells in the presence of the magnet was however similar to without magnet (25±6.5%). Similar to B16-F10 studies, the association of MAG-C electroneutral liposomes (with 2.5 mg/ml of MAG-C) significantly increased from 8.8±1.87% to 20.18±0.51% in presence of external magnet (Fig. 8).

3.10. Tumor accumulation of MAG-C cationic liposomes in presence of external magnet

In this study we examined the effect of the external magnet (1.2 T) on biodistribution of MAG-C cationic liposomes in B16-F10 melanoma bearing mice. First, the external magnet was placed on the external surface of the tumor followed by systemic injection of ^{111}In labeled MAG-C cationic liposomes. The magnet remained associated with the tumor mass for 1 h. The percent of label recovered by the tumor following exposure to external magnet was compared to the (unexposed) control group. At the 1 h time point we observed no significant difference between the two groups, as determined by the similar percent of label recovered by tumors in the presence (4.2±1.08) and absence (4.71±0.4) of magnet (Fig. 9). We however, did observe a significant difference at the 2 h time point (or 1 h following the removal of magnet). At this time interval tumors with no previous exposure to external magnet retained significantly less of the label, compared to tumors previously exposed to magnet ($P<0.05$; Fig. 9). Percent of label recovered by tumor in absence and presence of external magnet at 2 h time point was (5.04±0.73) and (3.3±0.54) respectively.

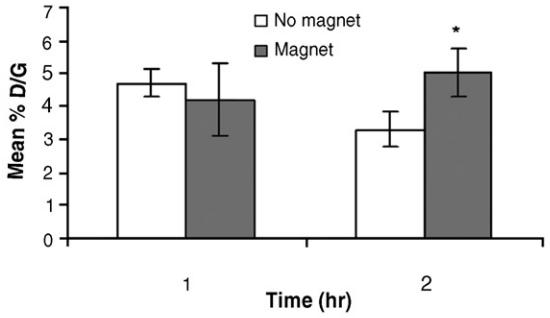


Fig. 9. Effect of external magnetic field on tumor distribution of MAG-C cationic liposomes. Approximately 2×10^6 B16-F10 cells were injected subcutaneously in mice bearing melanoma tumors of approximately 250 mm^3 volume. Mice were injected with ^{111}In labeled MAG-C cationic liposomes and external magnet of strength 1.2 T was placed for 1 h on the external surface of tumor mass. After 1 and 2 h post injection, radioactivity in tumor was measured; data are expressed as percent of label recovered per/ gram of tumor (see Experimetal procedures). Each value represents mean±S.D. of 4 animals, $P<0.05$.

The experimental findings suggest that the external magnet significantly enhanced tumor retention and facilitated vascular uptake of MAG-C cationic liposomes. Enhanced tumor vascular association is the preferred mechanism of uptake given the natural affinity of cationic liposomes for tumor vessels [13,14,28].

4. Discussion

The systemic administration of chemotherapeutic agents is a popular treatment approach used to control local tumor growth and distant metastasis, but not without some controversy. Today, there is an urgent need to reduce drug-associated side effects while preserving the efficacious nature of all chemotherapeutic agents. The field of nanotechnology has made remarkable strides in this direction. For example, the attachment of surface bound polymers (i.e., PEG) provide adequate protection of liposomes from unfavorable interactions with insoluble proteins in blood; such interactions alter tissue distribution and tumor-specific uptake [25,27].

In general, relatively long circulating liposomes result in enhanced delivery of drugs to solid tumors; however, the inclusion of PEG in preparations alone will not promote vascular-specific uptake in tumors. Earlier studies with cationic liposomes confirmed organ-specific uptake and preferential accumulation in tumors, specifically along tumor angiogenic vessels [13,28]. In order to preserve both the well-established benefits of PEG and the natural attraction of cationic liposomes for tumor vessels, PEG-modified cationic liposomes (PCLs) were evaluated in vivo [14]. Studies revealed preferential tumor vascular uptake but the distribution was non-uniform [14].

Fig. 8. Association of magnetic liposomes with melanoma and endothelial cells in presence of external magnet (0.4 T, exposure time—1 h) in vitro: Cells were seeded at 4×10^4 cells/ml in a 12 well plate and incubated at 37 °C. The percent association of cells with each liposome preparation type was determined 24 h following cell exposure to rhodamine labeled MAG-C PCLs and MAG-C electroneutral liposomes (500 nmol). The control group was untreated. Each value represents the mean±S.D. of 4 different determinations. (A) B16-F10, (B) HMEC-1.

In our experiments we set out to develop and characterize relatively tumor vascular-specific liposomes capable of responding to an external magnet. A potential candidate must demonstrate the ability to incorporate magnetic materials, maintain a mean diameter of around 150 nm, preserve a cationic charge potential, and avidly associate with human cancer and endothelial cells in vitro and in vivo.

We also investigated the role of PEG in our MAG-C cationic liposomes. The goal here was to develop relatively long circulating cationic liposomes that would permit additional time (if needed) for liposomes to respond to an externally applied magnet. All together, we report on the development and characterization of different components of bilayer physical properties of magnetic cationic liposomes. We determined the overall benefit of applying an external magnet. This was first accomplished by studying the interaction of magnetic cationic liposomes with melanoma and immortalized endothelial cells, serving as in vitro models of the interstitial and vascular compartments respectively. Our investigations represent the very first step in the development process.

In the first phase of our studies we evaluated the effect of various components on (i) liposome size, (ii) surface charge density, and (iii) membrane fluidity. The inclusion of 50 mol% DMTAP in DMPC preparations reduced the mean diameter of liposomes; but, the apparent reduction in size was not significant ($P>0.05$). The inclusion of cholesterol (10 mol%) in DMPC/DMTAP (1:1) mixtures did however reduce the mean diameter to a significant extent. Upon visual inspection of stock preparations cholesterol appeared to also eliminate unwanted liposome aggregates, resulting in a more homogenous stock preparation compared to DMPC/DMTAP alone. The ability to alter bilayer fluid dynamics, and increase bilayer stability are additional known effects of cholesterol [15]. In a previously reported study the effect of the inclusion of DOTAP (50 mol%) on liposome size of DMPC liposomes was investigated [18]. Computational studies carried out by Zuidam and Barenholz with DOPC/DOTAP (1:1) mixtures showed that both the quaternary amine of DOTAP and the phosphate group of DOPC are oriented in the same plane of the bilayer relative to the glycerol backbone [29]. In this arrangement the formation of a salt bridge might assist in the reduction of liposome size, in addition to exerting additional physical effects [18]. Our studies are consistent with the experimental and computational findings of these reports. We therefore speculate that the significant reduction in mean diameter of DMPC/DMTAP preparations in the presence of 10 mol% cholesterol was due to cholesterol's ability to elimination liposome aggregates, and DMTAP's ability to promote interactions between head group moieties of DMPC and DMTAP. When MAG-C (0.5 mg/ml) was included as part of the DMPC/ DMTAP/cholesterol preparation, we observed no additional change in size, however, an increase in MAG-C content (from 0.5 to 2.5 mg/ml) did increase liposome size ($P<0.05$).

The second bilayer property investigated (also critical to overcoming tumor vascular heterogeneity) was the effect of each component on cationic charge potential. Although cationic liposomes preferentially target the tumor endothelium, 50 mol% cationic lipid content was significantly more effective than 10 mol% [14]. Given that relatively high cationic charge density is necessary to achieve optimal vascular targeting efficiency, we determined to what extent each of our magnetic liposome components influence cationic charge potential.

The inclusion of 50 mol% DMTAP to DMPC liposomes was found to increase zeta potential values significantly. When MAG-C was added, the zeta potential value for DMPC/DMTAP/cholesterol preparations was significantly lower compared to same lipid composition without MAG-C. Given that the inclusion of MAG-C significantly reduced zeta potential of DMPC/DMTAP/cholesterol liposomes, its ability to alter bilayer properties involving surface charge is primarily due to the anionic charge of fluid MAG-C (−35 mv). In addition, MAG-C might well interact with the quaternary amine group of DMTAP, since DMPC and cholesterol are electroneutral and do not possess characteristics sufficient to shield a cationic charge density whether alone, or when used in combination. The findings suggest that MAG-C interacts with the (cationic) polar head group region of the bilayer, where it neutralizes some of the positive surface charge characteristics due to charge shielding. Zeta potential values for PCLs used to target tumor vessels were previously reported between 20 to 25 mv [14], suggesting that our values reported here for magnetic cationic liposomes are sufficient.The final bilayer property investigated involved effects on bilayer fluid dynamics. DPH polarization is a widely accepted method used to monitor changes in bilayer phase state due to the inclusion of various membrane constituents [19, 30]. DPH was used to determine how each liposome component exerts an effect on membrane fluidity. The phase transition temperature of DMPC liposomes was 26 °C; our findings were similar to values reported elsewhere [31,32]. The inclusion of DMTAP increased the phase transition temperature of DMPC liposomes from 26 to 42 °C, a significant increase in the fraction of liposome gel phase state. The addition of cholesterol further increased the phase transition temperature of DMPC/DMTAP liposomes, resulting in a bilayer that was apparently more rigid with considerably less acyl chain mobility. The phase transition temperature of DMPC/DMTAP/ cholesterol/MAG-C (0.5 mg/ml) liposomes was similar to cationic liposomes suggesting that MAG-C did not alter the bilayer physical properties at this concentration. However, MAG-C at a higher concentration (2.5 mg/ml) lowered the fluorescence polarization values of DMPC/DMTAP/cholesterol liposomes. So the overall extent to which MAG-C exerts an effect on liposome bilayer structure appears to be concentration dependent. Given the anionic charge potential (−35 mv) of MAG-C and the extent to which it exerts an effect on membrane fluidity, the sum of these interactions is probably not limited to the membrane interfacial region. Interactions may extend to include saturated acyl chains of DMPC and DMTAP as well.

Our analysis of DPH polarization studies, including the potent effect of MAG-C on cationic charge potential, is further supported by our data demonstrating the direct association of MAG-C with cationic liposome membranes (Fig. 4A, ii). The data also show that the presence of MAG-C in the liposome

membrane did not prevent melanoma (B16-F10) cells from internalizing cationic liposomes (Fig. 4A, iii).

Sulforhodamine B (SRB) assay is a method used to evaluate drug-induced cytotoxic effects in vitro [20]. SRB is a bright pink aminoxanthene dye used to stain basic proteins of viable cells. The successful staining of cellular proteins is a frequently used indicator of cellular viability [20]. We used the SRB assay to determine relative effect of different amounts of MAG-C cationic liposomes on murine (B16-F10) and human (HTB-72) melanoma, and human dermal endothelial (HMVEC-d) cells in vitro. We observed that <1 μmol of magnetic cationic liposomes was a suitable working concentration range, above which significant cell death was observed (data not shown).

We evaluated the interaction of magnetic cationic liposomes with B16-F10, HTB-72 and HMVEC-d cells both quantitatively and qualitatively. Our quantitative analysis of cultured cells revealed similar interactions of MAG-C cationic liposomes with B16-F10, HTB-72 and HMVEC-d.

We next determined the effect of 5% DMPE-PEG on the bilayer physical properties of magnetic cationic liposomes. We observed that the inclusion of PEG exerted no additional effects on liposome size (containing low MAG-C content), but significantly reduced zeta potential ($P<0.01$). However, DMPE-PEG significantly reduced liposome size (when relatively high MAG-C content was included). The reduction may be due to the stabilizing effect of PEG [33,34].

PEG reduced interaction of MAG-C preparations with all three cell lines. The polymer will reduce liposome uptake by the RES, but the extended circulation time provided by PEG in vivo may also improve overall tumor vascular uptake so long as the external magnet is applied. The interaction between cancer and endothelial cells with magnetic cationic liposomes was similar. Given the high affinity of tumor vessels for cationic liposomes and that tumor vessel have much greater access to systemically administered formulations, using magnetic PCLs for tumor vascular targeting (over interstitial delivery) is preferred.

When human endothelial cells were exposed to MAG-C cationic liposomes we observed no significant advantage of using the external magnet in vitro. We hypothesized that the naturally high affinity of mammalian cells for cationic liposomes (without the magnet) was responsible for the apparent lack of benefit. To confirm this hypothesis we used a low cellular binding electroneutral liposome variety to determine if the advantage of applying the external magnet could be observed under these conditions. We observed a significant increase in cell association in the presence of the magnet when 2.5 mg/ml of MAG-C was used, but not with 0.5 mg/ml. The avid electrostatic interactions initially observed between endothelial cells and MAG-C cationic liposomes were therefore responsible for the lack of benefit. More importantly, the amount of MAG-C included in liposome preparations will likely determine how well magnetic carriers respond to an external magnet.

In vitro screening of potentially useful magnetic formulations is both a time-sensitive and cost effective approach however, optimization may be necessary to achieve desired results. In addition to natural cell attractions in absence of magnet, other factors to consider are cell seed number and liposome amount. Although we observed no benefit of using magnetic cationic liposomes *in vitro*, we did however observe a benefit *in vivo*. Our experimental findings now support the use of MAG-C cationic liposomes as a carrier for the delivery and prolonged tumor accumulation of chemotherapeutic agents in the presence of an external magnetic field.

The use of magnetic liposomes has been demonstrated for various applications [4,7,10,17,35,36]. Nobuto et al., showed that administration of magnetic DOX liposomes with subsequent application of magnetic field (0.4 T) produced a 3 to 4 fold higher doxorubicin concentration in tumors of osteosarcoma bearing hamsters, compared to magnetic DOX liposomes without external magnet [17]. Zhang et al., extended the circulation half life of paclitaxel and decreased uptake by RES with the administration of lyophilized paclitaxel magneto liposomes also under the influence of external magnet [4].

In conclusion, our data suggest that MAG-C cationic liposomes satisfy an important set of criteria. Our liposomes can incorporate MAG-C, maintain a cationic charge potential in presence of magnetic material, are taken up by cancer and endothelial cells, and under certain conditions can respond to an external magnet in vitro and in vivo. These characteristics are necessary to overcome heterogeneous vascular targeting with cationic liposomes when used to induce tumor vascular injury with vascular disrupting agents. For this reason subsequent in vivo investigations are warranted.

Acknowledgments

The authors thank Dr. Vladimir Torchilin for kindly providing access to the Beckmann Gamma counter, and members of his group (Drs. Tamer El-Bayoumi and Tatyana Levchenko) for assistance with the biodistribution experiment. We finally thank Chemicell in Berlin Germany for providing fluid MAG-C. The manuscript represents work submitted in partial fulfillment of the Doctoral Degree in Pharmaceutical Sciences, Northeastern University, Boston, MA (for S.D.).

References

[1] H.C. Pitot, Cancer—An overview, in: E.R. Lafond (Ed.), Cancer: The Outlaw Cell, 2nd ed., American Chemical Society, Washington, 1988, pp. 1–18.
[2] D.J. Chaplin, G.J. Dougherty, Tumour vasculature as a target for cancer therapy, Br. J. Cancer 80 (Suppl. 1) (1999) 57–64.
[3] C. Alexiou, A. Schmid, R. Klein, P. Hulin, C. Bergemann, W. Arnold, Magnetic drug targeting: biodistribution and dependency on magnetic field strength, J. Magn. Magn. Mater. 252 (2002) 363–366.
[4] J.Q. Zhang, Z.R. Zhang, H. Yang, Q.Y. Tan, S.R. Qin, X.L. Qiu, Lyophilized paclitaxel magnetoliposomes as a potential drug delivery system for breast carcinoma via parenteral administration: in vitro and in vivo studies, Pharm. Res. 22 (2005) 573–583.
[5] C. Alexiou, W. Arnold, R.J. Klein, F.G. Parak, P. Hulin, C. Bergemann, W. Erhardt, S. Wagenpfeil, A.S. Lubbe, Locoregional cancer treatment with magnetic drug targeting, Cancer Res. 60 (2000) 6641–6648.
[6] C. Alexiou, R. Jurgons, R.J. Schmid, C. Bergemann, J. Henke, W. Erhardt, E. Huenges, F. Parak, Magnetic drug targeting-biodistribution of the magnetic carrier and the chemotherapeutic agent mitoxantrone after locoregional cancer treatment, J. Drug Target. 11 (2003) 139–149.

[7] M. Babincova, V. Altanerova, C. Altaner, P. Cicmanec, P. Babinec, In vivo heating of magnetic nanoparticles in alternating magnetic field, Med. Phys. 31 (2004) 2219–2221.
[8] K.J. Widder, A.E. Senyei, D.F. Ranney, Magnetically responsive microspheres and other carriers for the biophysical targeting of antitumor agents, Adv. Pharmacol. Chemother. 16 (1979) 213–271.
[9] A.S. Lubbe, C. Bergemann, J. Brock, D.G. McClure, Physiological aspects in magnetic drug-targeting, J. Magn. Magn. Mater. 194 (1999) 149–155.
[10] M. Babincova, V. Altanerova, M. Lampert, C. Altaner, E. Machova, M. Sramka, P. Babinec, Site-specific in vivo targeting of magnetoliposomes using externally applied magnetic field, Z. Naturforsch, (C) 55 (2000) 278–281.
[11] R. Kunstfeld, G. Wickenhauser, U. Michaelis, M. Teifel, W. Umek, K. Naujoks, K. Wolff, P. Petzelbauer, Paclitaxel encapsulated in cationic liposomes diminishes tumor angiogenesis and melanoma growth in a "humanized" SCID mouse model, J. Invest. Dermatol. 120 (2003) 476–482.
[12] S. Strieth, M.E. Eichhorn, B. Sauer, B. Schulze, M. Teifel, U. Michaelis, M. Dellian, Neovascular targeting chemotherapy: encapsulation of paclitaxel in cationic liposomes impairs functional tumor microvasculature, Int. J. Cancer 110 (2004) 117–124.
[13] G. Thurston, J.W. McLean, M. Rizen, P. Baluk, A. Haskell, T.J. Murphy, D. Hanahan, D.M. McDonald, Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice, J. Clin. Invest. 101 (1998) 1401–1413.
[14] R.B. Campbell, D. Fukumura, E.B. Brown, L.M. Mazzola, Y. Izumi, R.K. Jain, V.P. Torchilin, L.L. Munn, Cationic charge determines the distribution of liposomes between the vascular and extravascular compartments of tumors, Cancer Res. 62 (2002) 6831–6836.
[15] F. Szoka Jr., D. Papahadjopoulos, Comparative properties and methods of preparation of lipid vesicles (liposomes), Annu. Rev. Biophys. Bioeng. 9 (1980) 467–508.
[16] T. Kubo, T. Sugita, S. Shimose, Y. Nitta, Y. Ikuta, T. Murakami, Targeted systemic chemotherapy using magnetic liposomes with incorporated adriamycin for osteosarcoma in hamsters, Int. J. Oncol. 18 (2001) 121–125.
[17] H. Nobuto, T. Sugita, T. Kubo, S. Shimose, Y. Yasunaga, T. Murakami, M. Ochi, Evaluation of systemic chemotherapy with magnetic liposomal doxorubicin and a dipole external electromagnet, Int. J. Cancer 109 (2004) 627–635.
[18] R.B. Campbell, S.V. Balasubramanian, R.M. Straubinger, Influence of cationic lipids on the stability and membrane properties of paclitaxel-containing liposomes, J. Pharm. Sci. 90 (2001) 1091–1105.
[19] R.B. Campbell, S.V. Balasubramanian, R.M. Straubinger, Phospholipid-cationic lipid interactions: influences on membrane and vesicle properties, Biochim. Biophys. Acta 1512 (2001) 27–39.
[20] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, J. Natl. Cancer Inst. 82 (1990) 1107–1112.
[21] L. Ricotti, A. Tesei, F. De Paola, P. Ulivi, G.L. Frassineti, C. Milandri, D. Amadori, W. Zoli, In vitro schedule-dependent interaction between docetaxel and gemcitabine in human gastric cancer cell lines, Clin. Cancer Res. 9 (2003) 900–905.
[22] T.S. Levchenko, R. Rammohan, A.N. Lukyanov, K.R. Whiteman, V.P. Torchilin, Liposome clearance in mice: the effect of a separate and combined presence of surface charge and polymer coating, Int. J. Pharm. 240 (2002) 95–102.
[23] V.P. Torchilin, Liposomes as targetable drug carriers, Crit. Rev. Ther. Drug Carr. Syst. 2 (1985) 65–115.
[24] K.J. Harrington, C.R. Lewanski, J.S. Stewart, Liposomes as vehicles for targeted therapy of cancer. Part 1: preclinical development, Clin. Oncol. (R. Coll. Radiol.) 12 (2000) 2–15.
[25] T. Ishida, H. Harashima, H. Kiwada, Liposome clearance, Biosci. Rep. 22 (2002) 197–224.
[26] S.N. Sarkar, S.V. Balasubramanian, S.K. Sikdar, Effect of fenvalerate, a pyrethroid insecticide on membrane fluidity, Biochim. Biophys. Acta 1147 (1993) 137–142.
[27] V.P. Torchilin, V.S. Trubetskoy, which polymers can make nanoparticulate drug carriers long-circulating? Adv. Drug Delivery Rev. 16 (1995) 141–155.
[28] J.W. McLean, E.A. Fox, P. Baluk, P.B. Bolton, A. Haskell, R. Pearlman, G. Thurston, E.Y. Umemoto, D.M. McDonald, Organ-specific endothelial cell uptake of cationic liposome–DNA complexes in mice, Am. J. Physiol. 273 (1997) H387–H404.
[29] N.J. Zuidam, Y. Barenholz, Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin, Biochim. Biophys. Acta 1329 (1997) 211–222.
[30] B.R. Lentz, Use of fluorescent probes to monitor molecular order and motions within liposome bilayers, Chem. Phys. Lipids 64 (1993) 99–116.
[31] D. Needham, E. Evans, Structure and mechanical properties of giant lipid (DMPC) vesicle bilayers from 20 degrees C below to 10 degrees C above the liquid crystal-crystalline phase transition at 24 degrees C, Biochemistry 27 (1988) 8261–8269.
[32] R. Zantl, L. Baicu, F. Artzner, I. Sprenger, G. Rapp, J.O. Radler, Thermotropic phase behaviour of cationic lipid–DNA complexes compared to binary lipid mixtures, J. Phys. Chem., B 103 (1999) 10300–10310.
[33] C.M. Lee, Y. Choi, E.J. Huh, K.Y. Lee, H.C. Song, M.J. Sun, H.J. Jeong, C.S. Cho, H.S. Bom, Polyethylene glycol (PEG) modified 99mTc-HMPAO-liposome for improving blood circulation and biodistribution: the effect of the extent of PEGylation, Cancer Biother. Radiopharm. 20 (2005) 620–628.
[34] O. Garbuzenko, Y. Barenholz, A. Priev, Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer, Chem. Phys. Lipids 135 (2005) 117–129.
[35] M. Babincova, P. Sourivong, D. Chorvat, P. Babinec, Laser triggered drug release from magnetoliposomes, J. Magn. Magn. Mater. 194 (1999) 163–166.
[36] M. Babincova, P. Cicmanec, V. Altanerova, C. Altaner, P. Babinec, AC-magnetic field controlled drug release from magnetoliposomes: design of a method for site-specific chemotherapy, Bioelectrochemistry 55 (2002) 17–19.