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The effect of monosialylganglioside mix modifying the PEGylated liposomal epirubicin on the accelerated blood clearance phenomenon

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ABSTRACT

PEGylated liposomes are potential candidates to improve the pharmacokinetic characteristics of encapsulated drugs, to extend their circulation half-life and facilitate their passive accumulation at tumour sites. However, PEG-modified liposomes can induce accelerated blood clearance (ABC) upon repeated administration, and the extent of ABC phenomenon on the cytotoxic drugs-containing PEGylated liposomes is related to the dose of the cytotoxic drugs. In this study, EPI served as a model cytotoxic drug, a hydrophilic surfactant molecule, monosialylganglioside (GM1) was chosen and modified on the liposomes together with PEG. It was shown that upon mixed modification, when GM1 contents reached 10% or 15% mol, the ABC phenomenon of the PEGylated liposomal EPI significantly reduced. We also found that GM1 played an important role in abrogating the ABC phenomenon in both the induction phase and the effectuation phase. The results suggested that GM1 incorporation unfortunately did not avoid occurrence of ABC phenomenon completely, but GM1 modification on PEGylated liposomes may provide a significant improvement in clinical practice of PEGylated liposomes. Further study must be necessary.

1. Introduction

Liposomes, representing the clinically approved nanomedicine systems, are well-characterized, simple, and versatile platforms for the manufacture of functional and tuneable drug carriers [1,2]. However, it was found that conventional liposomes underwent massive uptake readily by the mononuclear phagocyte system (MPS), which limited the targeting effect of drugs to the therapeutic sites *in vivo* [3]. PEGylated nanocarriers are potential candidates to improve the pharmacokinetic characteristics of encapsulated drugs and to extend their circulation

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half-life for better therapeutic efficacy [4]. Nevertheless, Dams et al. [5] reported that when PEG-modified liposomes were repeatedly injected intravenously in the same animal (with an interval of several days), it resulted in the loss of the long circulating characteristic and extensive accumulation in the liver and spleen, and this phenomenon is called "accelerated blood clearance (ABC) phenomenon".

Interestingly, the repeated administration of Doxil®, a PEGylated liposomal formulation of the cytotoxic drug doxorubicin (DXR), within the dose range of 10–60 mg/m^2 in clinical practice did not induce the ABC phenomenon [6], which may be due to the drug's cytotoxicity on splenic marginal zone B cells. However, Suzuki et al. [7] recently reported that Doxil®, at lower doses (less than 2 mg DXR/m^2), induced severe ABC phenomenon in the beagle dog. Upon repeated administration of DXR-containing PEGylated liposomes, different results were attributed to the amount of anti-PEG IgM production. The IgM producers, proliferating splenic B cells, were inhibited and/or killed because of the toxicity of DXR released from the first injected liposomes.

Meanwhile, a novel treatment regimen of sequential low-dose chemotherapy, termed "metronomic" chemotherapy, has been broadly applied, in particular, when combined with radiation therapy and anti-angiogenic agents in recent years [8–10]. In contrast with conventional chemotherapy, this method dramatically reduces the toxic side effects, and decreases the risk of multidrug resistance by tumour cells [11]. Our group previously examined the effect of the sequential administration of PEGylated liposomal epirubicin (EPI) on the induction of the ABC phenomenon. We demonstrated that the first or sequential injections of PEGylated liposomal EPI within a certain range (0.08–1.20 mg epirubicin/kg) in Wistar rats induced an instant clearance of subsequently injected PEGylated liposomal EPI, leading to the abolishment of its therapeutic efficacy [12]. The induction of the ABC phenomenon upon repeated administration of PEGylated liposomes will hinder the development of sequential low-dose regimen. For cytotoxic drugs, the ABC phenomenon could cause cumulative damage in the MPS organs such as the liver and spleen. These results mean that the ABC phenomenon will exist or cannot be negligible when the lower dose of the cytotoxic drugs containing PEGylated liposomes are administrated, which will alter the pharmacokinetics of the liposomal preparations *in vivo*.

The mechanism that induces the ABC phenomenon has been extensively evaluated, but is still unclear. At present, the ABC phenomenon is divided into two phases, the induction phase and the effectuation phase. During the induction phase, the first injection of PEGylated liposomes acts as TI-2 antigens and induces an immunological response by tightly cross-linking to the cell surface immunoglobulins of specific B cells, resulting in increased anti-PEG IgM production [13,14]. In the effectuation phase, following the second injection, the produced anti-PEG IgM selectively binds with the PEG on the liposomes, and subsequently activates the complement system, which in turn leads to opsonization by C3 fragments, and enhances the uptake by Kupffer cells endocytosis [15,16]. Meanwhile, recent studies also have shown that the connecting portion between the hydrophilic PEG chain and the hydrophobic segment of the PEG-conjugates such as PEG–distearoylphosphatidylethanolamine (PEG–DSPE), may be the key site for anti-PEG IgM to distinguish, which mediates the ABC [17].

Considering the above factors, we could choose a hydrophilic surfactant molecule and modify it on the liposomes together with PEG. On one hand, the mixed modification would regulate the neat conformation of the PEG moiety on the surface of the PEGylated liposomes, resulting in disturbing the production of anti-PEG IgM in the induction phase. On the other hand, the hydrophilic surfactant molecule could mask the hydrophobic connecting portion of the PEG–DSPE on the modified liposomes in the effectuation phase, and thereby, abrogate or attenuate the ABC phenomenon.

Monosialylganglioside (GM1) is a widely used hydrophilic surfactant molecule, which has inherent biodegradability and non-immunogenicity and is found on cell membrane *in vivo* [18]. GM1 consists of a hydrophobic "tail", inserted in the membrane lipid bilayer, and a hydrophilic "head" of oligosaccharide sugar, mainly including the D-glucose (Glc), D-galactose (Gal), N-acetylgalactosamine (GalNAc), N-acetylneuraminic acid (NeuNAc, sialic acid), and N-hydroxy-acetylneuraminic acid [18,19]. Several studies have attempted to explore the immune camouflage potential of GM1. In order to increase the blood circulation time, liposomes were coated with GM1 to imitate erythrocytes [20]. Gabizon and Papahadjopoulos [21] reported that GM1-modified liposomes induced a 3.4-fold decrease in the MPS uptake compared with conventional liposomes after intravenous injection in mice. Liu et al. [22] showed that GM1-modified liposomes could escape MPS uptake. Thus, GM1, a non-immunogenic and biodegradable material, may have the potential function to abrogate or attenuate the ABC phenomenon of the PEGylated formulation.

In the present study, EPI served as a model cytotoxic drug, a commonly used anthracycline antitumour antibiotic, and GM1, at various molar concentrations, was modified on the PEGylated liposomes encapsulating EPI. The purpose was to verify the effect of GM1 on abrogating or attenuating the ABC phenomenon caused by PEGylated liposomes, such as their pharmacokinetic behaviour and tissue distribution after the second injection in rats. The findings may provide a solution to the undesirable immune response, and have a great significance in clinical practice of PEGylated liposomes.

2. Materials and methods

2.1. *Materials*

Monosialylganglioside (GM1) was purchased from Chongqing Huanrui Biology Technology Co, Ltd (Chongqing, China). Epirubicin·HCl (EPI, purity 99.0% by high-performance liquid chromatography) was received from Olympic Star Pharmaceutical Co, Ltd (Shenzhen, China). Hydrogenated soy phosphatidylcholine (HSPC) was obtained from Lucas Meyer (Düsseldorf, Germany). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (mPEG2000–DSPE) was purchased from Shanghai Advanced Vehicle Technology Pharmaceutical Ltd (Shanghai, People's Republic of China). Cholesterol (CH) was obtained from China National Medicines Co, Ltd (Shenyang, China). ZB-1 cation exchange fibres were acquired from Guilin Zhenghan Tech-

Table 1 – Compositions and characterizations of the prepared liposomes.

Formulation	Compositions	Size (nm)	*Zeta* potentials (mV)	Entrapment efficacy (%)
EPI-PL(0.0% mol GM1)	HSPC:CH:mPEG2000–DSPE:GM1 (3:1:1:0.0, wt/wt)	109.1 ± 1.6	–28.1 ± 2.1	97.4 ± 3.1
EPI-PL(2.5% mol GM1)	HSPC:CH:mPEG2000–DSPE:GM1 (3:1:1:0.3, wt/wt)	106.8 ± 7.4	–32.1 ± 3.4	96.2 ± 2.4
EPI-PL(5.0% mol GM1)	HSPC:CH:mPEG2000–DSPE:GM1 (3:1:1:0.6, wt/wt)	100.9 ± 1.4	–33.4 ± 1.9	97.2 ± 3.6
EPI-PL(10% mol GM1)	HSPC:CH:mPEG2000–DSPE:GM1 (3:1:1:1.2, wt/wt)	101.8 ± 3.5	–38.1 ± 3.7	95.1 ± 4.2
EPI-PL(15% mol GM1)	HSPC:CH:mPEG2000–DSPE:GM1 (3:1:1:1.8, wt/wt)	105.5 ± 2.3	–39.5 ± 3.6	95.3 ± 1.8

nology Development Co, Ltd (Guilin, China). All other chemicals used in this study were of analytical or HPLC grade.

2.2. *Animals*

The male Wistar rats with the age of 7–8 weeks (weight, 180–220 g) were purchased from the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All rats had free access to food and water. The animal care and experiments were performed in accordance with the guidelines of the local Animal Welfare Committee and Guide for the Care and Use of Laboratory Animals [23].

2.3. *Preparation of liposomes*

Liposomes were prepared by using the modified ethanol injection method [12,24]. Briefly, the lipid mixture (see Table 1) was dissolved in absolute ethanol and then evaporated at 65 °C to near dryness. The resulting dry lipid film was hydrated at 65 °C for 20 min in citrate buffer (pH 4.0) under mechanical agitation to achieve a final lipid concentration of 50 mg/ml. After hydration, the obtained multilamellar vesicles (MLV) was sonicated using a laboratory ultrasonic cell pulverizer (JY92-II; Ningbo Scientz Biotechnology Co, Ltd, Ningbo, China) for at least one 2-min cycle (200 W) and an additional 6-min cycle (400 W). The suspension was then successively filtered through 0.80-, 0.45-, and 0.22-μm filter membranes at 25 °C to remove large particles. The mean diameters and *zeta* potentials of liposomes were determined by NICOMP 380 HPL submicron particle analyser (Particle Sizing System, CA, USA).

2.4. *Drug loading and determination of encapsulation efficiency (EE)*

EPI was loaded into liposomes by using a pH gradient method. Briefly, a transmembrane pH gradient (ΔpH = 3) was established by adding sodium phosphate solution (0.5 M) into the suspension of empty liposomes. Subsequently, EPI solution (4 mg/ml) and empty liposomes were mixed at a ratio of 1:10 (*wt*/*wt*) between the drug and lipid, and then the mixture was incubated for 20 min at 60 °C.

After the drug was loaded, the untrapped EPI was removed using a cation exchange fibre column to determine the encapsulation efficiency (EE). Briefly, 100 μl of samples were loaded onto a 732 cation exchange resin mini-column (10 × 25 mm) and then eluted with distilled water. The eluate was solubilized with 90% (*v*/*v*) isopropyl alcohol containing 0.75 mol/l HCl, and the concentration of EPI in the liposomal samples was assessed photometrically at 480 nm (UV-1801 UV/VIS spectrophotometer; Beijing Rayleigh Analytical Instrument Co, Ltd, Beijing, China). The per cent EE was calculated as the percentage of EPI remaining in the liposomes following elution.

2.5. In vitro *release assay*

EPI solution or loaded liposomes were transferred to dialysis bags. Drug release experiments were performed by dialysis against 120 ml of the release buffer (300 mM xylitol, 80 mM NH_4Cl and 10 mM histidine, pH 7.4). A volume of 1 ml of EPI solution or loaded liposomes was placed in dialysis bag (10 KDa MWCO) at 37 °C. At various time points, aliquots were withdrawn and stored at –20 °C until analysis. The concentration of EPI was measured using a microplate reader fluorescence spectrophotometer (Molecular Devices Ltd., USA). The emission/excitation wavelengths of EPI were 502/562 nm.

2.6. *Pharmacokinetics and biodistribution of liposomes* in vivo

Male Wistar rats were randomly divided. For the first injection, the Wistar rats were initially administered with GM1 molar modified PEGylated liposomal EPI via the tail vein at a dose of 1 μmol phospholipids/kg or 5% glucose solution instead of the liposomes. Seven days later, the serum samples were collected 30 minutes before the second injection. For the second injection, GM1 modified PEGylated liposomal EPI was injected intravenously at a dose of 5 μmol phospholipids/kg. At 0.083, 0.25, 0.5, 1, 2, and 4 h following intravenous injection, blood samples were collected in a volume of 250 μl by eye puncture. After withdrawing the last blood sample at 4 hours, the liver and spleen were excised, rinsed in ice-cold normal saline, and snap frozen. Blood samples were centrifuged at 4000 rpm for 10 min to isolate the plasma. The plasma and tissue samples were stored at –20 °C until assayed. The detailed injection scheme is presented in Table 2.

To investigate the effect of GM1 on the PEGylated liposomes in the induction phase and the effectuation phase, the detailed injection scheme is presented in Tables 3 and 4, respectively.

2.7. *Analytical procedure*

To monitor the *in vivo* pharmacokinetic behaviour of liposomes, the concentrations of EPI in plasma and tissue samples were assayed by using a spectrofluorometric method. The tissues were homogenized by using a Tissue-Tearor equipped with an 80 mm probe (IKA WORKS GUANGZHOU, Guangzhou, China). Before EPI analysis, the samples were treated as follows:

Table 2 – The injection scheme of the various liposomes.

Groups	First injection (1 μmol phospholipids/kg)	Second injection (5 μmol phospholipids/kg)	ABC index$_{(0–30\ min)}$
A1	5% Glucose injection	EPI-PL(0.0% mol GM1)	/
B1	5% Glucose injection	EPI-PL(2.5% mol GM1)	/
C1	5% Glucose injection	EPI-PL(5.0% mol GM1)	/
D1	5% Glucose injection	EPI-PL(10.0% mol GM1)	/
E1	5% Glucose injection	EPI-PL(15.0% mol GM1)	/
A2	EPI-PL(0.0% mol GM1)	EPI-PL(0.0% mol GM1)	0.067 ± 0.01
B2	EPI-PL(2.5% mol GM1)	EPI-PL(2.5% mol GM1)	0.083 ± 0.02
C2	EPI-PL(5.0% mol GM1)	EPI-PL(5.0% mol GM1)	0.120 ± 0.01
D2	EPI-PL(10.0% mol GM1)	EPI-PL(10.0% mol GM1)	0.621 ± 0.05
E2	EPI-PL(15.0% mol GM1)	EPI-PL(15.0% mol GM1)	0.584 ± 0.07

3.9 and 3.8 ml extraction solutions (methanol/water, 50/50 containing 0.3 M HCl, *v*/*v*) were added to 0.1 ml plasma and 0.2 ml tissue homogenate, respectively. The mixture was then vortexed for 5 min, and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to another 7.0 ml-Eppendorf tube, and centrifuged at 10,000 rpm for another 10 min. The final supernatant was added into 96-well plates and assayed using a microplate reader fluorescence spectrophotometer (Molecular Devices Ltd., USA). The emission/excitation wavelengths of EPI were 502/562 nm.

2.8. *Detection of anti-PEG IgM antibodies*

mPEG2000–DSPE, at a concentration of 0.2 mmol/l, was added to the wells of a 96-well plate (Corning Incorporated, New York, USA) and the coated plate was thoroughly air dried. Tris-buffered saline (50 mM) containing 0.14 mM NaCl and 1% bovine serum albumin (BSA) was added to block the wells, and the plate was incubated for 60 min. After incubation, the wells were washed 3 times with 0.05% CHAPS in Tris-buffered saline (pH 8.0). Then, 100 μl of the diluted serum samples (1% BSA, 0.05% CHAPS, 50 mM Tris, pH 8.0) were added into the 96-well plate, which was incubated for 60 min and washed 5 times as described above. Horseradish peroxidase (HRP)-conjugated antibody (100 μl, 1 μg/ml, rabbit anti-rat IgM–HRP conjugate) was added to each well, and the wells were washed 5 times after incubation for 60 min as described above. The coloration was initiated by using 100 μl of o-phenylenediamine (1 mg/ml, Sigma–Aldrich, St. Louis, MO, USA). After the wells were incubated for 15 min, the reaction was stopped by the addition of 100 μl 2N sulphuric acid (H_2SO4). The absorbance was measured at 490 nm by using a microplate reader fluorescence spectrophotometer (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The experiment was performed at room temperature.

2.9. *Statistical analysis*

All data are presented as the mean ± standard deviation. Statistical comparisons were performed using Student's *t*-test with SPSS 17.0 software. *P*-values lower than 0.05 were considered statistically significant.

Table 3 – The injection scheme of GM1 on PEGylated liposomes in the induction phase.

Groups	First injection (1 μmol phospholipids/kg)	Second injection (5 μmol phospholipids/kg)	ABC index$_{(0–30\ min)}$
PL-PL	EPI-PL(0.0% mol GM1)	EPI-PL(0.0% mol GM1)	0.067 ± 0.01
2.5GPL-PL	EPI-PL(2.5% mol GM1)	EPI-PL(0.0% mol GM1)	0.096 ± 0.03
5.0GPL-PL	EPI-PL(5.0% mol GM1)	EPI-PL(0.0% mol GM1)	0.128 ± 0.01
10.0GPL-PL	EPI-PL(10.0% mol GM1)	EPI-PL(0.0% mol GM1)	0.628 ± 0.08
15.0GPL-PL	EPI-PL(15.0% mol GM1)	EPI-PL(0.0% mol GM1)	0.591 ± 0.03

Abbreviation: PL, EPI-PL (0.0% mol GM1); 2.5GPL, EPI-PL (2.5% mol GM1); 5.0GPL, EPI-PL (5.0% mol GM1); 10.0GPL, EPI-PL (10.0% mol GM1); 15.0GPL, EPI-PL (15.0% mol GM1).

Table 4 – The injection scheme of GM1 on PEGylated liposomes in the effectuation phase.

Groups	First injection (1 μmol phospholipids/kg)	Second injection (5 μmol phospholipids/kg)	ABC index$_{(0–30\ min)}$
PL-PL	EPI-PL(0.0% mol GM1)	EPI-PL(0.0% mol GM1)	0.067 ± 0.01
PL-2.5GPL	EPI-PL(0.0% mol GM1)	EPI-PL(2.5% mol GM1)	0.095 ± 0.04
PL-5.0GPL	EPI-PL(0.0% mol GM1)	EPI-PL(5.0% mol GM1)	0.192 ± 0.02
PL-10.0GPL	EPI-PL(0.0% mol GM1)	EPI-PL(10.0% mol GM1)	0.301 ± 0.01
PL-15.0GPL	EPI-PL(0.0% mol GM1)	EPI-PL(15.0% mol GM1)	0.523 ± 0.06

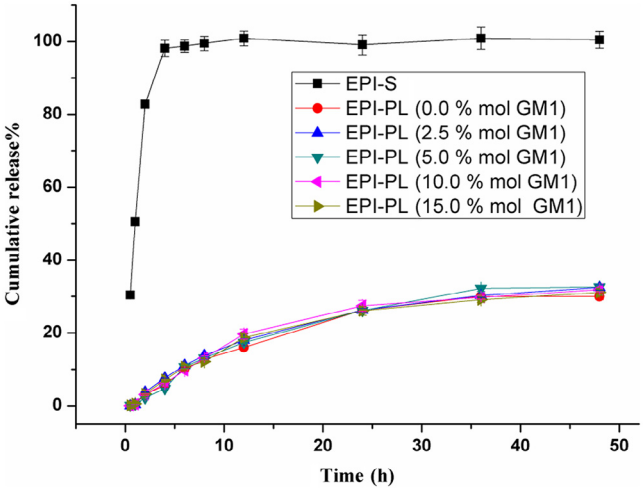

Fig. 1 – *In vitro* release of PEGylated liposomal EPI containing various molar GM1 modifications. Date represented as Mean ± SD, *n* = 3.

3. Results and discussion

3.1. *The characteristics of the liposomes*

In the present study, liposomal EPI was prepared by a pH gradient. The mean particle sizes of the prepared liposomes were 90–110 nm. *Zeta* potentials demonstrated that all the liposomes were negatively charged, from –20 to –40 mV. Moreover, for all formulations, the per cent encapsulation efficiency (%EE) of EPI in the prepared liposomes was over 95%. Details with regard to the pharmaceutical properties of the prepared liposomes are summarized in Table 1.

3.2. In vitro *release assay*

As seen in Fig. 1, *in vitro* release experiments, free EPI could completely permeate the dialysis bag at 6 h, while only a small amount of drug leaked from the liposomal preparations. In addition, there was no significant difference in drug release rate among various GM1 molar content modified PEGylated liposomal EPI ($P > 0.05$). This result suggested EPI stayed stable in the liposomes and was suitable a marker for studying the pharmacokinetics of liposomal preparations.

3.3. *Pharmacokinetics of the first intravenous injection of PEGylated liposomal EPI containing various GM1 molar modifications*

The rats were administrated with the PEGylated liposomal EPI containing various GM1 molar modifications by intravenous injection via the tail vein at a dose of 5 μmol phospholipids/kg, and the pharmacokinetics was determined by using a spectrofluorometric method. Fig. 2A showed that the circulation time of the GM1 modified PEGylated liposomal EPI was a little shorter than that without GM1. On one hand, it may be ascribed to the changed conformation of the PEGylated liposomes due to the addition of GM1. For a 100 nm-liposomal particle grafted with DSPE–PEG2000, PEG chains should be arranged in the mushroom mode in the presence of <4 mol% DSPE–PEG2000, while in the transition mode with a 4–8 mol% modification, and in the brush mode with >8 mol% PEGylation [25,26]. Thus, in the present study, depending on the 5 mol% DSPE–PEG2000 modification, brush and mushroom mode transition states would have coexisted, which is expected to be the most stable state, and prevented opsonin uptake [27]. GM1, the hydrophilic surfactant molecule, would convert the coexisting brush and mushroom mode transition state to brush mode alone in space conformation, or increase the ratio of brush mode in the two coexisting states, resulting in the reduction in compliance on the liposome surface. On the other hand, after GM1 modified on PEGylated liposomes, the head of this molecule surfactants would take up part of the surface of the liposomes, which would lower the coverage of PEG. GM1 consists of four monosaccharides, and the compliance and hydrophilicity of the component oligosaccharide chains are lower than that of DSPE–PEG2000 chain. The two factors contributed to the weaker regulatory effect of PEGylated liposomes with GM1

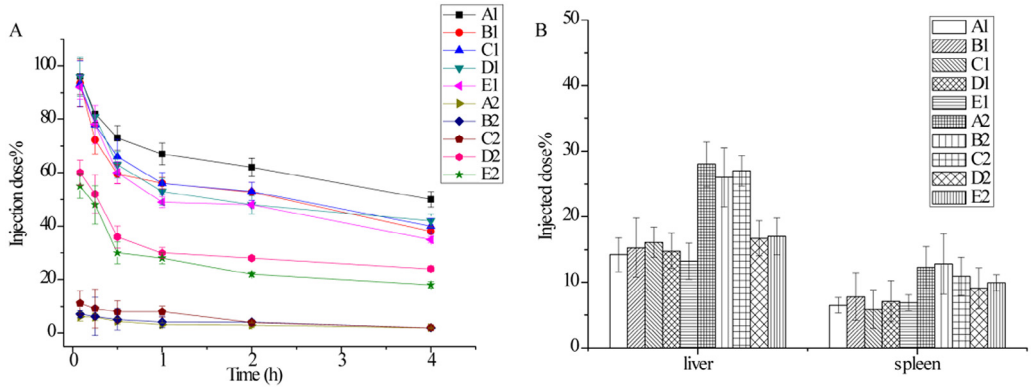

Fig. 2 – The pharmacokinetic and tissue distribution of PEGylated liposomal EPI containing various molar GM1 modifications. From groups A1 to E1 represented pre-dosing with 5% glucose injection, while A2 to E2 represented the effect of repeated injection of PEGylated liposomal EPI containing various molar GM1 modifications on the ABC phenomenon in rats. (A) Blood clearance. (B) Hepatic and splenic accumulation 4 h after intravenous injection of single injection and repeated injection. Data are shown as Mean ± SD, *n* = 3.

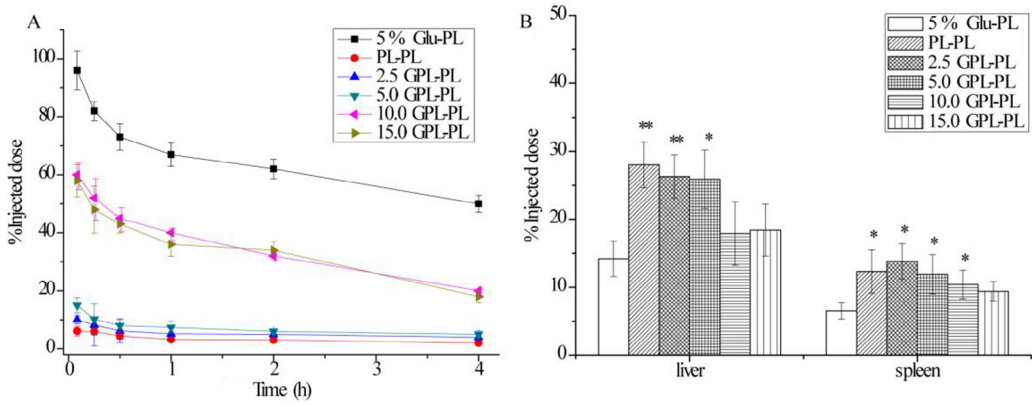

Fig. 3 – Effect of GM1 on PEGylated liposomal EPI in the induction phase of the ABC phenomenon in rats. The control represents no pre-dosing; 7 days later, the rats were given PEGylated liposomal EPI without GM1 modification. (A) Blood clearance. (B) Hepatic and splenic accumulation 4 h after intravenous injection of the test dose. Data are shown as Mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with 5% Glu-PL.

modification than without GM1 modification, thus corresponding to a shorter circulation time *in vivo*. Dexi Liu has introduced that rats have naturally occurring anti-GM1 antibody which induces rapid clearance of GM1-modified liposome [28]. The presence of such antibody would make complication on understanding the results in my study. So in rats, single and repeated only GM1-modified liposome would not change the circulation time and uptake by the MPS respectively in the study (data not shown), which proved that there were no anti-GM1 antibodies in the rats we had used in this study.

3.4. *Effect of repeated injection of PEGylated liposomal EPI containing various molar GM1 modifications on the ABC phenomenon*

The effect of GM1 with various molar content modifications on the PEGylated liposomal EPI on inducing the ABC phenomenon after repeated injection was investigated. As illustrated in Fig. 2A and 2B, the GM1 molar concentrations of the PEGylated liposomes on the first injection were 2.5%, 5.0%, 10.0%, and 15.0%, respectively. Although, all the prepared liposomes caused the ABC phenomenon in the repeated intravenous injection, there were significant differences in the extent of the ABC phenomenon. When the molar concentrations of GM1 were 2.5% and 5.0%, the EPI concentrations in the blood decreased significantly at the first time point after injection, which presented severe ABC phenomenon and there was no significant difference in the comparison with liposomes without GM1. When the GM1 molar concentration reached 10.0% and 15.0%, the EPI concentrations in the blood decreased slowly and the ABC phenomenon of the second injection reduced by about 50%, compared with the PEGylated liposomal EPI alone ($P < 0.05$).

3.5. *Effect of GM1 on the PEGylated liposomal EPI in the induction phase of the ABC phenomenon*

To investigate the effect of GM1 on PEGylated liposomes in the induction phase of the ABC phenomenon, the rats were administered with various GM1 molar content modified PEGylated liposomal EPI via the tail vein at a dose of 1 μmol phospholipids/kg as the first injection. After 7 days, for the second injection, PEGylated liposomal EPI was injected intravenously at a dose of 5 μmol phospholipids/kg. As shown in Fig. 3A, when the modified GM1 content reached 10% and 15% mol, the EPI in PEGylated liposomes decreased slowly in circulation *in vivo* after the second injection. Compared to PL-PL group (Fig. 3B), the accumulation of EPI in hepatic and splenic significantly decreased ($P < 0.05$).

3.6. *Effect of GM1 on the PEGylated liposomal EPI in the effectuation phase of the ABC phenomenon*

In order to survey the role of GM1 modifications on PEGylated liposomal EPI in the effectuation phase, the rats were administered intravenously with PEG-modified liposomes as the first injections. After 7 days, PEGylated liposomal EPI containing various molar GM1 modifications served as second injection. As shown in Fig. 4A and 4B, with the increasing GM1 content in the second injections, the plasma pharmacokinetic profile of EPI gradually slowed down. When the content was 15%, its pharmacokinetic profile and hepatosplenic distribution presented the most significant difference ($P < 0.05$, *compared with PL-PL*).

3.7. *Comparison of the ABC index of the GM1 modified PEGylated liposomal EPI following repeated injections*

Ishihara et al. [29] used the ABC index, the ratio of the area under the concentration–time curve from time 0 to the last measured time point (AUC_{0-t}) for the second injection to that of the first injection, as a parameter to evaluate the ABC phenomenon. A higher index indicates a slower clearance of PEGylated nanocarriers from the blood circulation and a weaker induction of the ABC phenomenon. In this study, the ratio of $AUC_{(0-30\ min)}$ was adopted to assess the ABC phenomenon. We calculated the ratio for different periods (0–30 min, 0–1 h, 0–2 h, and 0–4 h), which showed that the discrepancies in the ratio

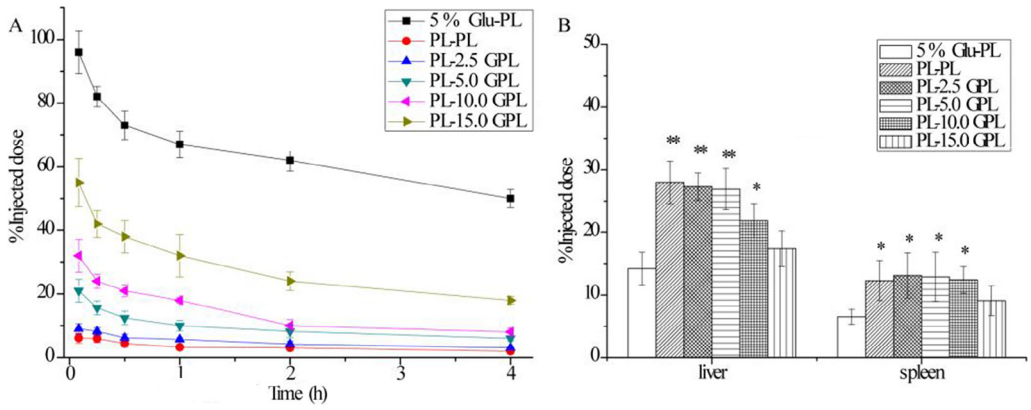

Fig. 4 – Effect of GM1 on PEGylated liposomal EPI in the effectuation phase of the ABC phenomenon in rats. The control represents no pre-dosing; 7 d later, the rats were given PEGylated liposomal EPI without GM1 modification. (A) Blood clearance. (B) Hepatic and splenic accumulation 4 h after intravenous injection of the test dose. Data are shown as Mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with 5% Glu-PL.

did not change (data not shown). The results were similar to the previous study in our group [30]. With the view that the accelerated blood clearance effect lasted only a short time, so the ABC index$_{(0–30\ min)}$ was applied to evaluate the extent of the ABC phenomenon. Table 2 demonstrated an increase in the ABC index$_{(0–30\ min)}$ after different intravenous injection schemes. As seen from the table, with the various GM1 molar modifications on PEGylated liposomal EPI on repeated injections, the ABC index$_{(0–30\ min)}$ was the highest to the lowest as follows: 15.0% GM1 modification > 10.0% GM1 modification > 5.0% GM1 modification > 2.5% GM1 modification. As demonstrated, when the GM1 modification reached 10.0% and 15.0%, the ABC index$_{(0–30\ min)}$ increased significantly ($P < 0.05$). In the study, the ABC phenomenon in the induction phase showed a decrease in the following order: 15.0GPL-PL > 10.0GPL-PL > 5.0GPL-PL > 2.5GPL-PL > PL-PL. In the study, the ABC phenomenon in the effectuation phase, showed a decrease in the following order: PL-15.0GPL > PL-10.0GPL > PL-5.0GPL > PL-2.5GPL > PL-PL.

In the present study, after repeated injection of PEGylated liposomal EPI, the administered dose of EPI was 0.08 mg per kg by the 1 μmol phospholipids/kg as the first injection, which would cause severe ABC phenomenon. As seen from the acquired plasma curve drug and ABC index, when the concentration of GM1 reached to 10% and 15% mol in the repeated injections of liposomes, the ABC phenomenon significantly reduced. The results may be closely related to the characteristics of GM1. GM1 is an amphipathic substance, consists of a hydrophilic oligosaccharide chains and lipophilic ceramide, wherein a sialic acid moiety is attached to the oligosaccharide chains. Based on the accepted mechanism of the ABC phenomenon, in the induction phase, PEGylated liposomal EPI used in the first injection, which is regarded as T-cell independent type 2 antigens (TI-2), stimulated the splenic marginal zone B cells to produce anti-PEG IgM, resulting in the accelerated blood clearance of subsequently administered liposomes. In general, most TI-2 antigens with neat surface arrangements are large multivalent molecules that exhibit a prolonged circulation time *in vivo* [16]. Based on the common property of TI-2 antigens, the interaction between the antigens and membrane receptors in B cells and persistent B-cell signalling induced an immune response after the repeated injection. The lipophilic portions of GM1 are inserted within the phospholipid bilayer, and the hydrophilic oligosaccharide chains on the surface of the bilayer on the PEGylated liposomes. When the GM1 content modification on PEGylated liposome surface reaches a certain level, the interaction between the oxygen atom of the PEG chain and the hydroxyl group of sialic acid on GM1 would result in changes in conformation of PEGylated EPI liposomes, which would disrupt the TI-2 antigen characteristics. As introduced above, GM1 consists of four monosaccharides, including sialic acid. It has been reported that GM1 can reduce the immunogenicity of the modified liposomes [31]. Michalek et al. [31] reported that GM1, when incorporated into the activating liposome membrane at molar ratios between 10^{-2} and 10^{-5}, inhibited activation in a dose-dependent manner. They presented data that sialic acid is a very efficient inhibitor of the alternative pathway of human C, showing activity at molar concentrations as low as 0.01%. In the present study, when the GM1 content modification on PEGylated EPI liposomes surface reached 10 and 15% mol, the conformation of liposomes would change, meanwhile a portion of GM1 would be exposed to the liposome surface, which may reduce the immunogenicity of the modified liposomes, and thus weaken the ABC phenomenon on the repeated injection. While the data that GM1 reduced immunogenicity of the modified liposomes to reduce the ABC phenomenon was not sufficient, the mechanism needed further study.

3.8. *Comparison of anti-PEG IgM produced by the PEGylated liposomal EPI containing various GM1 molar modifications*

It has been reported that there is a positive correlation between anti-PEG IgM production and the strength of the ABC phenomenon caused by PEGylated liposomes [32]. To investigate whether the GM1 would influence the production of anti-PEG IgM, the anti-PEG IgM level in the serum was assessed on day 7 after the initial intravenous dose of PEGylated liposomes

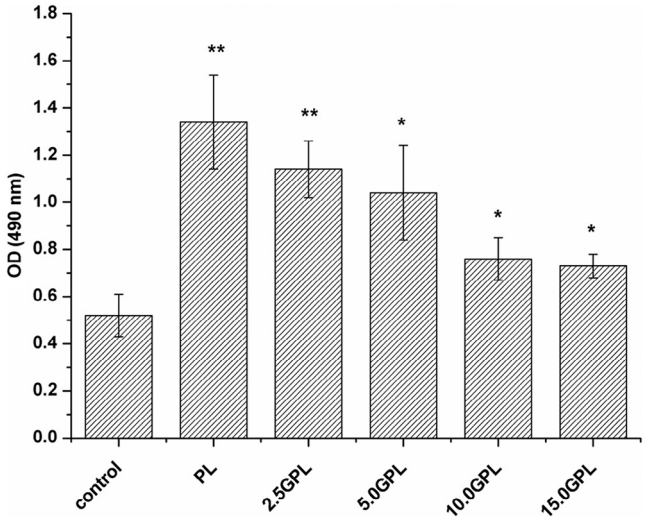

Fig. 5 – Anti-PEG IgM production induced by the initial priming dose of PEGylated liposomes containing various GM1 modifications after intravenous injection. The anti-PEG IgM in the serum was determined using ELISA. Data are shown as Mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group.

containing various GM1 modifications. Accordingly, in this study, the IgM antibody reactive to the PEG on the PEGylated liposomes was quantized using classical ELISA [33–35]. As illustrated in Fig. 5, the absorbance ratio in the rank order of the highest to the lowest was as follows: 2.5% GM1 modification > 5.0% GM1 modification > 10.0% GM1 modification > 15.0% GM1 modification. The results indicated that the induction of the ABC phenomenon was accompanied by an increase in the anti-PEG IgM level.

The production of anti-PEG IgM was detected following the first injection of the different GM1 modifications on the PEGylated liposomes. We found that with the increase content modification of GM1, the production of anti-PEG IgM reduced, which also verified the above view that GM1 would disturb the neat conformation, and disrupt the TI-2 antigen characteristics. Recent studies also have shown that in PEG-conjugates such as DSPE–PEG2000, the connecting portion between the hydrophilic PEG chain and the hydrophobic segments may be the key sites for anti-PEG IgM to recognize PEG modified formulations. Yokoyama et al. [17] revealed that anti-PEG IgM seemed to recognize the interface between the hydrophilic PEG chain and hydrophobic block of PEG-conjugates, which mediated the accelerated blood clearance, rather than recognize the PEG main chain. We chose GM1 modification on the liposomes with PEG, which masked the hydrophobic connecting portion of PEG–DSPE on the modified liposomes. Then PEGylated liposomal EPI was injected in rats as the first injection, and various GM1 molar content modifications on PEGylated liposomal EPI were intravenously administrated to verify the effect of GM1 in the effectuation phase. As illustrated in Fig. 4A and 4B, the rats acquired the same amount of anti-PEG IgM for the first injection *in vivo*, and after injection of GM1 modified PEGylated liposomes, the ABC phenomenon reduced with increasing GM1 modification. The results demonstrated that GM1 could mask the hydrophobic connecting portion of PEG–DSPE on the modified liposomes, which would attenuate the ABC phenomenon.

4. Conclusion

In this study, GM1 with various molar concentrations was modified on the PEGylated liposomal EPI, and the effect of GM1 on abrogating or attenuating the ABC phenomenon of PEGylated liposomal EPI was investigated, such as their pharmacokinetic behaviour and tissue distribution after the second dose in Wistar rats. Results showed that when GM1 contents reached 10% or 15% mol, the ABC phenomenon of the PEGylated liposomal EPI significantly reduced. We also found that GM1 played an important role in abrogating the ABC phenomenon in both the induction phase and the effectuation phase. GM1 modification on PEGylated liposomes may provide a significant improvement in clinical practice of PEGylated liposomes.

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