Synthesis and Application of Methoxy Poly(ethylene glycol)-Bile Salts Conjugates in Physicochemical Characterization and the Pharmacokinetics of the Liposomal Bifendate in Rats

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ABSTRACT: Stealth liposomes have been broadly investigated as drug delivery or diagnostic agent. However, the materials that possess the ability of stealth, such as DSPE-PEG and Chol-PEG, are either costly or synthetic complex. In this research with different molecular weights (2000–6000 g/mol) of methoxy poly(ethylene glycol)(MePEG), a series of MePEG-bile (MePB) conjugates were generated by an economical and simple method and confirmed by FTIR and ^{1}H-NMR spectrum. The properties in aqueous solution were studied, including viscosity and surface activity, over a wide concentration range. To elucidate the application of MePB in liposomes (MePBL), conventional liposomes (CL) were prepared, and the influence of the grafting density and the chain length of MePB in liposomes were investigated. The ability of long circulation of MePBL was evaluated by intravenous injection administration in rats. Results indicated that all the liposomes prepared, with or without MePB composition, were similar in micrograph, and the contents of MePB in MePBL were more important than the chain length of MePB for a long circulation *in vivo*.
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Key words: stealth liposome; methoxy (polyethylene glycol)-bile salts; DDB; sustained release; pharmacokinetics

INTRODUCTION

It is well known that liposome is able to improve the therapeutic index of a variety of drugs. Intravenously administered liposomes generally undergo extensive opsonization and are therefore rapidly cleared by macrophages of the mononuclear phagocyte system (MPS), particularly Kupffer cells in the liver and spleen macrophages. This drawback could be amended by coating liposomes with hydrophilic, neutral polymers such as polyethylene glycol (PEG).[1] It has been reported that PEG could form a hydrated membrane around the liposome surface and increase the hydrophilicity of the liposome.[2] Therefore, these PEGylated lipoplexes or polyplexes could decrease the interaction of liposome with opsonins[3–5] and prolong the time of liposome *in vivo*.

Previously, scientists had synthesized various PEG derivatives, such as DSPE-PEG, Chol-PEG, and so on. Besides PEG, several other hydrophilic polymers have been successfully applied to coat the long-circulating liposome, among them conjugates based on poly(oxazoline),[6] polyglycerol,[7] poly(N-(2-hydroxypropyl) methacrylamide)),[8] poly-N- vinylpyrrolidone,[9,10] and polyvinyl alcohol.[11] However, all these materials are either costly or synthesizes complex that limited its application seriously.

The objective of this research is to find a novel material that is not only providing liposomes with long circulation time as compared with conventional liposomes (CLs) but also have the advantage of being easily synthesized. It is well known that the cholesterol is one of the content of the liposome. Therefore, scientists conjugated the cholesterol and PEG through the butanedioic anhydride. And the cholesterol insert in the bilayer of the liposome, the PEG exposes in outside form a hydrated membrane around the liposome surface and so increase the hydrophilic of the liposome. The structure of bile acid and the cholesterol are extremely similar (Fig. 1), but the bile acid may carry on the response directly with PEG, thus the reduction process of synthesis reduces the synthesis cost. So we first

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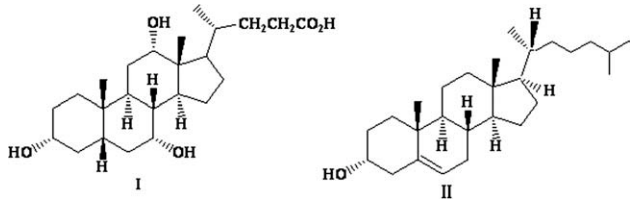

Figure 1 The structure of Bile (I) and Chol (II).

synthesized a novel polymer MePB by bile acid and MePEG ($MePEG_{2000}$, $MePEG_{4000}$, and $MePEG_{6000}$) direct response and confirmed by FTIR, ^{1}H-NMR, and investigated the physicochemical properties. The physicochemical characteristics of MePB surface-modified liposomes (MePBL) and CLs were prepared and determined within this study. At last, to examine the feasibility of MePB coating CL to achieve sustained drug delivery system, we carried out integral animals to study the pharmacokinetics profiles of CL and MePBL. And we also studied the influence of the $MePEG_{2000}$-Bile (MeP_2B) grafting density and the chain length in liposomes on the circulation kinetics of MePBL.

Bifendate (DDB), which is widely used in China, could lower alanine transaminase (ALT) in patients.[12–14] Because of poor solubility in water, there are only oral preparations on market that results in low bioavailability. However, for acute hepatitis patients and those with decreased liver functions after surgical operations parenteral dosages would offer the best benefits for them. For this purpose, several groups had tried to improve the solubility of DDB in water and prepare the DDB solution for intravenous injection.[15] In our previous studies, the $t_{1/2\beta}$ of DDB liposome was about 108.99 min. To prolong the time of DDB *in vivo*, we modified the CL with MePB. The pharmacokinetics of DDB entrapped in MePBL and CL of rats were also evaluated.

EXPERIMENTAL

Characterization

IR spectra of succinyl bile, MePEG, and the reaction products were recorded on a Bruker FTIR Tensor 27 spectrophotometer (KBr disk). ^{1}H-NMR was performed on a 300-MHz Bruker NMR spectrometer using $CDCl_3$ as a solvent.

Measurements of the steady state viscosity were performed on a Brookfield viscometer (Brookfield DV-III) with Rheocak v2.7 data manager. The measurement was carried out at 20°C with various angular velocities. A typical reaction was performed within a shear rate range of 50–350 s^{-1}. The device registered viscosity values every 20 s.

The surface activity of bile, $MePEG_{2000}$, and MePB conjugates had been established by performing a systematic tensiometric study in aqueous solution at concentration from 10^{-5} to 10 mg/mL.

The determination of size, zeta potential, and polydispersity index (PI) of the liposomes were performed by dynamic light scattering (Zetasizer 3000HSA, Malven Instruments BK). The shape of the liposomes was observed by the transmission electron microscope (H-7000 Hitachi, Japan) at an accelerating voltage of 75 kV. DDB concentration was determined by measurement of absorbance at 278 nm (SPD-20A, Shimadzu Co., Tokyo, Japan) after dissolving the liposomes in methanol.

Materials and methods

DDB (99.0% purity) was supplied by Zhejiang Hisoar Pharmaceutical Company. MePEG with M_n 2000, 4000, and 6000 g/mol was purchased from the Xilong Chemical Reagent Corp. Bile salt was purchased from Sigma-Aldrich chemical. Soybean phosphatidylcholine (PC) was purchased from Shanghai Taiwei Pharmaceutical Industry (Shanghai, China, PC > 92%). Cholesterol (China Medicine Shanghai Chemical Reagent Corp., China) and succinic anhydride (Shanghai Chemical Reagent Co., China) were used as received. *N*, *N*-dicyclohexylcarbodiimide (DCC) was obtained from Sinopharm Chemical Reagent Co. The chemical, 4-dimethylaminopyridine (DMAP), was purchased from the Zhejiang Xianju Pharmaceutical and Chemical Experimental Plant. All other reagents were of AR.

Rats (male, 180–220 g) were purchased from the Experimental Animal Center of China Pharmaceutical University and housed in groups of four under standard laboratory conditions and had free access to rat chow and water. All animal experiments were performed according to national regulations and approved by the local animal experiments ethical committee.

Synthesis of MePB

According to our previously studies,[16] the synthesis of MePB involved two steps of chemical modification on bile salts by esterification, one with acetic anhydride to obtain acetyl bile ester, and then with MePEG to obtain sufficiently hydrophilic terminal.

Liposome preparation

The preparation of MeP_2B and CL were according to a modification of the method of Bangham et al.[17] The MePB, PC, and Chol were dissolved in 10-mL ethanol, and the solution was dried to form a thin film, and the film was hydrated with a 5% glucose solution to make the liposome suspension. The liposome of $MePEG_{2000}$-Bile (MeP_2BL) that contained different content of MeP_2B ($MeP_2B/PC = 3\%$, 5%, and 8%) was

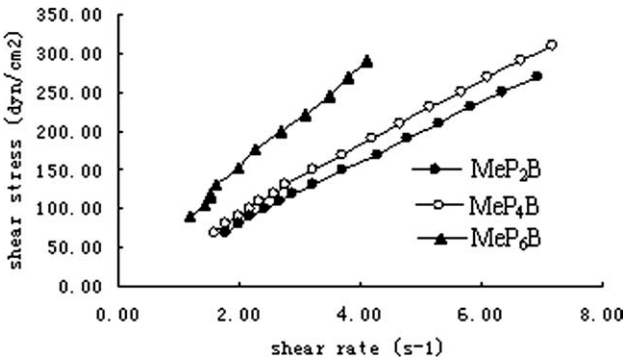

Figure 2 Shear stress versus shear rate for 100.0 ug/mL MePB solution in distilled water at 20°C.

prepared as described earlier for the purpose of evaluating the influence of the MeP_2B grafting density in liposomes on the circulation kinetics of MeP_2BL. To evaluate the effect of the chain length of MePB on the circulation kinetics of liposome, we prepared difference chain length liposome using MeP_2B, $MePEG_{4000}$-Bile (MeP_4B), and $MePEG_{6000}$-Bile (MeP_6B).

Physicochemical properties of CL and MePBL

In this study, the encapsulation efficiency (EE) of the liposomes was determined according to the published method.[18] In brief, a 0.5 mL of DDB liposomes suspension was added into a dialysis membrane bag. The encapsulation efficiency was calculated according to the following equation:

$$\mathrm{EE}\% = \left(1 - \frac{M_{\mathrm{free}}}{M_{\mathrm{total}}}\right) \times 100\%,$$

where M_{total} is the total amount of DDB in DDB liposomes, and M_{free} represents the amount of free DDB not encapsulated in the liposomes.

Pharmacokinetic in blood of CL and MePBL

Forty eight rats were divided randomly into eight groups. CL or different of MePBL were respectively injected into rats (0.9 mg/kg) via tail vein at a single dose. After intravenous injection, the rats were anesthetized with aether, and a heparinized capillary was then inserted into the eyeground veins to get 0.3-mL blood into a plastic centrifuging tube at the time intervals of 5, 10, 15, 30, 45, 60, 90, 120, 240, and 480 min, respectively. All samples were immediately frozen at −20°C until analysis.[19]

RESULTS AND DISCUSSION

Synthesis and characterization of MeP_2B

The product was purified by column chromatography on silica gel H using dichloromethane/methanol (50 : 1, v/v) as an eluent. A single spot by TLC analysis $R_f = 0.70$ (CH_2Cl_2 : methanol : acetic acid = 10 : 1 : 0.05) was visualized with iodine vapor. To further detect whether esterification occurred, the FTIR and ^{1}H-NMR spectra of triacetyl bile ester, $MePEG_{2000}$, and MeP_2B were measured. Compared with 3 acetyl bile ester, the FTIR spectra of MeP_2B appears the new peak in 1344, 1242, 1117, and 843 cm^{-1}. The peak that appears in 1117 cm^{-1} belongs to the stretching vibration of C—O—C of $MePEG_{2000}$. The ^{1}H-NMR spectrum appears the new peak in δ (ppm) 3.38(s, 3H, OCH_3), 3.50–3.78(m, 148H), which belong to the —CH_2CH_2O—, (—OCH_3) of the $MePEG_{2000}$. All these results further indicated that the esterification had definitely occurred. The MeP_4B and MeP_6B were synthesized as described earlier.

Rheological studies

The shear stress–shear rate relationship of MePB dispersion in distilled water was shown in Figure 2, which shown a typical Newtonian viscosity behavior. The Herschel–Bulkley model[20] took account of both the yield stress and the shear thinning behavior of MePBL conjugates and fitted the experimental data very well (correlation coefficient exceeded 0.99 for all conjugates prepared). Therefore, this model was appropriate to describe the rheological properties for the phenomenon of shear thinning observed. Shear thinning results from the tendency of the applied force to disturb the long chains from their favored conformation, causing elongation in the direction of shear.[21] Although PEG was a nonionic hydrophilic chain, the presence of the bile group may result in hydrophobic associations.

Surface tension data

Figure 3 shows the surface tension of bile, $MePEG_{2000}$, and different MePB conjugate dispersion. Addition of the bile in the water decreased the surface tension value about 33 dyn/cm (mN/m)

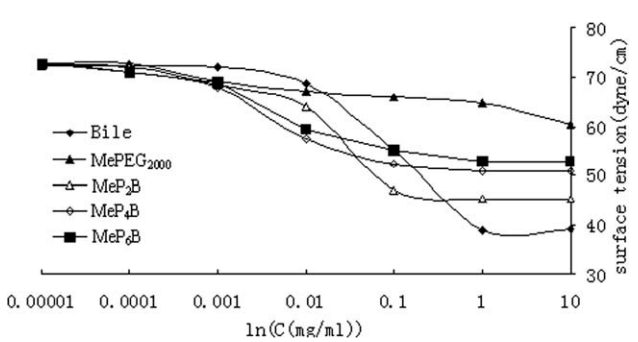

Figure 3 Plots of surface tension as a function of the bile, $MePEG_{2000}$, and MePB conjugates concentration for MePB at 20°C.

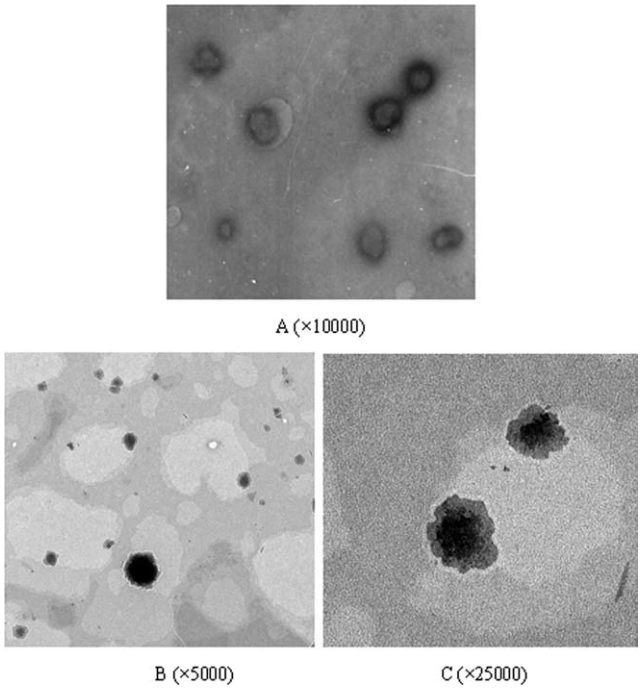

Figure 4 Transmission electron microscope photographs of liposomes (A), MeP_2B-LP (B, C).

compared with pure water, while the presence of the $MePEG_{2000}$ had no apparently changed. From the results, we could also know that MePB with different long chain had a variation surface activity. These trends were in line with the macromolecular nature of the conjugates. In fact, the surface activity of the MePB could be attributed to its weak amphiphilic nature, while the bile tail confers to the conjugate a very hydrophobic character. The chain of MeP_6B was the longest that may be in particular had higher flexibility that made it difficulty to align on the surface, and so had the highest surface tension. As it could be seen from the surface tension plateau values, the ability to decrease the surface tension was brought about by the equilibrium of its surface-active analytes comprising both hydrophobic and hydrophilic structural regions. Thus, in aqueous solution, these species diffused toward the air/liquid interface and were preferentially adsorbed at the surface, thereby lowering the surface tension of the solution.[22,23]

Physicochemical properties of CL and MePBL

The physicochemical properties of CL and MePBL were studied. As shown in Figure 4, the shape of liposome was round or oval by the transmission electron microscope. From Table I, we could see that the contents of MeP_2B did not affect the EE of MeP_2BL remarkably. And the particle size increased along with the increase in the contents of MeP_2B. On the contrary, the zeta potential and the PI were decreased along with the increase in the contents of MeP_2B. From these results, we might conclude that the hydration shell of liposome was increased. The effect of the chain length of MePB on the physicochemical properties of MePBL was shown in Table II. There was no markedly difference among MeP_2BL, the liposome of $MePEG_{4000}$-Bile (MeP_4BL), and the liposome of $MePEG_{6000}$-Bile (MeP_6BL), which means the chain length of MePB was not the important factor that affects the physicochemical properties of MePBL.

Pharmacokinetics of CL and MeP_2BL

The mean concentration–time profiles of CL and MeP_2BL in plasma were shown in Figure 5. All the curves were fit the open two-compartment model. And the major pharmacokinetic parameters were listed in Table II. The C_{max} value of MeP_2BL was 614.64 ± 89.68 ng/mL, which was significantly different from that of the CL (402.36 ± 34.35 ng/mL) ($P < 0.05$). The MRT and $T_{1/2\beta}$ of the MeP_2BL were increased compared with those of CL. The plasma AUC ratio of MeP_2BL to the CL was 175.68%. All these results implied that MeP_2B did increase the CL in plasma, retarded its clearance, and exhibited sustained-release properties *in vivo*.

The influence of the chain of MePB on the pharmacokinetics of MePBL

The effect of the chain of MePB (MeP_2B, MeP_4B, and MeP_6B) on the circulation kinetics of MePBL was shown in Figure 5, and the major pharmacokinetic parameters were listed in Table II. From the results, we could conclude that along with the increase of the chain length of the MePB, the $AUC_{0\text{-}T}$, $MRT_{0\text{-}T}$,

TABLE I
The Physicochemical Properties of Different Formulations ($n = 3$)

MeP_2B	EE (%)	Particle size (nm)	Zeta potential (mv)	PI
CL	90.65 ± 4.22	274 ± 36	−24.91 ± 2.03	0.542 ± 0..032
3% MeP_2B	86.89 ± 5.62	309 ± 19	−22.21 ± 4.36	0.447 ± 0.025
5% MeP_2B	88.65 ± 3.84	312 ± 28	−20.95 ± 3.86	0.369 ± 0.034
8% MeP_2B	85.76 ± 4.43	332 ± 13	−19.66 ± 5.33	0.325 ± 0.047
3% MeP_4B	89.14 ± 5.99	298 ± 32	−24.22 ± 2.55	0.381 ± 0.081
3% MeP_6B	81.49 ± 3.22	324 ± 25	−21.25 ± 5.06	0.395 ± 0.044

TABLE II
Pharmacokinetic Parameters of DDB After Intravenous Injection of Different Formulations in Rats ($n = 6$)

Compartmental parameters	Parameters values ± SD					
	CL	MeP_2BL	MeP_4B	MeP_6B	5% MeP_2B	8% MeP_2B
$MRT_{0\text{-}T}$ (min)	118.11 ± 28.61	198.86 ± 10.85	209.33 ± 18.47	221.91 ± 18.93	222.80 ± 10.86	249.20 ± 12.75[a]
$AUC_{0\text{-}T}$ (min ng/mL)	24832.78 ± 9334.50	43627.70 ± 3333.54[b]	48183.94 ± 3241.11	5024.66 ± 2847.37	70915.2 ± 416.95[a]	124259.90 ± 1042.87[a]
Cl ($\times 10^{-5}$ mg min^{-1} (ng/mL)$^{-1}$)	4.18 ± 0.26	2.0 ± 0.21[a]	1.7 ± 0.13	1.3 ± 0.10	1.2 ± 0.3	0.6 ± 0.02[a]
$T_{1/2\beta}$ (min)	108.99 ± 45.19	148.12 ± 47.77	159.09 ± 32.31	174.23 ± 40.35	162.18 ± 28.95	269.55 ± 19.66[a]
C_{max} (ng/mL)	402.36 ± 34.35	614.64 ± 89.68[a]	625.92 ± 74.24	702.42 ± 92.39	1485.90 ± 165.40[a]	2084.67 ± 215.92[c]

Values are expressed as mean ± s.d.
[a] Compared with 3%, MeP_2BL, $P < 0.05$.
[b] Compared with CL, $P < 0.05$.
[c] Compared with 3%, MeP_2BL, $P < 0.001$.

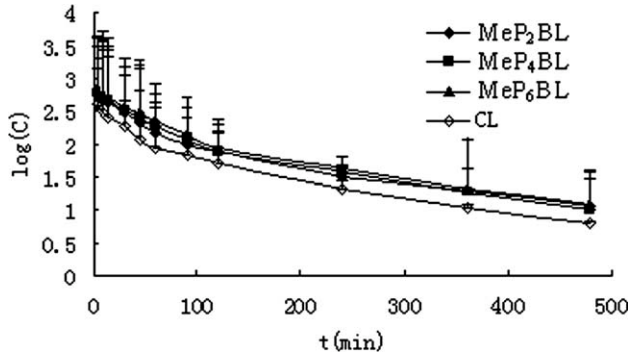

Figure 5 Mean plasma concentration profiles of different formulations after intravenous injection into rats ($n = 6$).

$T_{1/2\beta}$, and Cl of MEPBL were increased. However, there was no statistics difference among them, which mean that the chain length may not be an important factor in the design of long-circulating liposomes.

The influence of the MeP_2B grafting density on the pharmacokinetics of MeP_2BL

The effect of grafting levels of MeP_2B (i.e., 3%, 5%, and 8% MeP_2B) on the circulation kinetics of MeP_2BL was shown in Figure 6. The major pharmacokinetic parameters were listed in Table II. The results showed that there were no significant differences between 3% MeP_2BL and 5% MeP_2BL on $MRT_{0\text{-}T}$, $T_{1/2\beta}$, and Cl. However, the $AUC_{0\text{-}T}$ and Cmax was increased along with the increase of the content of MeP_2B. When the contents of MeP_2B in the MeP_2BL increased to 8%, the major pharmacokinetic parameters were remarkably different from those of 3% MeP_2BL. The $MRT_{0\text{-}T}$ was increased to 249.02 ± 12.75 min from 198.86 ± 10.85 min when the contents of MeP_2B increased from 3% to 8%. And the $AUC_{0\text{-}T}$, C_{max}, and $T_{1/2\beta}$ of 8% MeP_2BL were increased about 2.85, 3.39, and 1.82 times compared with that of 3% MeP_2BL, respectively. The Cl

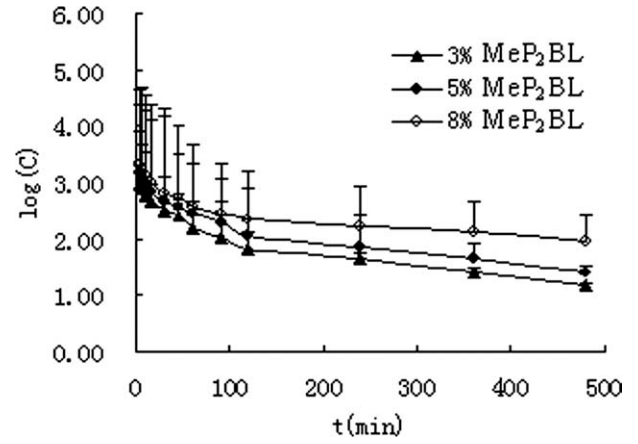

Figure 6 Mean plasma concentration profile of different MeP_2BL after intravenous injection into rats ($n = 6$).

was decreased to 1/3 times. This is in line with the findings on the effect of grafting density of MeP_2B on liposome circulation times.[24] The density of stealth polymers on the liposome surface had been shown to be of importance in the design of long-circulating liposomes.

CONCLUSIONS

In the present research, we have synthesized a series of novel MePEG-conjugated bile salt (MePB) by simple method and then successfully modified liposomes of DDB by using MeP_2B, MeP_4B, or MeP_6B, respectively. The rheological studies of MePB dispersion in distilled water were a typical Newtonian viscosity behavior and fitted the Herschel–Bulkley model very well. From the results, we also know that MePB with different long chain had a variation surface activity. These trends were in accord with the macromolecular nature of the conjugates. The results were shown that the grafting density could affect the properties of MeP_2BL and increase the hydration shell of liposome along with the increasing MeP_2B contents. On the contrary, the properties of MeP_2BL, MeP_4BL, and MeP_6BL were no markedly difference, which means that the chain length was not an important factor that affects the physicochemical properties of MePBL.

Then, we studied the pharmacokinetics of MeP_2BL and CL *in vivo*. From the results, we could know that the novel MeP_2B did delay CL clearance and exhibit sustained-release profile. The density of stealth polymers on the liposome surface had been shown to be of importance in the design of long-circulating liposomes. And in preclinical studies for PEG-liposomes with a PEG_{2000}-conjugate, a grafting density of 5 to 7.5 mol % was often used.[25,26] At PEG levels >4 mol %, a transition from the mushroom state, where PEG chains do not interact laterally, to the denser and thicker brush state of PEG occurs, the latter being the condition for optimal steric stabilization of the liposomes.[27] It has been shown that a further increase of the density of PEG up to 20 mol % did not change circulation kinetics and biodistribution. Our studies show that at grafting densities between 3% and 8%, MePB was able to prolong liposome circulation time *in vivo*. From the studies of the effect on the MePBL chain, we could conclude that the major pharmacokinetic parameters of MePBL were increased with the increasing MePB chain length. However, there was no statistics difference among them, which means that the chain length may not be an important factor for designing long-circulating liposomes. Taken together, these results suggested that MePB could be used to modify liposomes as a new drug carrier and possess a sustained-release profile.

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