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Abstract

PEGylated liposomes (PEG-Lip) have been widely used as a drug carrier for its good stealth property in blood circulation. However, the second injection of PEG-Lip was reported to result in ABC phenomenon and trigger hypersensitivity reactions in sensitive individuals for its complement activation effect. To avoid adverse immune responses, HA was selected to modify liposomes to afford HA modified liposomes (HA-Lip). Repeated administrations of PEG-Lip and HA-Lip were performed in rats. Our results showed PEG-Lip induced ABC phenomenon accompanied by a greatly increased accumulation of PEG-Lip in the liver. In contrast, HA-Lip showed good stealth property without inducing either the ABC phenomenon or an increase in liver uptake. Moreover, HA-Lip did not trigger the complement activation in human serum *in vitro* and in rat blood *in vivo*. Consequently, HA modification represents a viable strategy to prolong the blood circulation time of liposomes without inducing ABC phenomenon and adverse immune responses.

Key words: ABC phenomenon, complement activation, liposomes, hyaluronic acid

1. Introduction

Composing of a lipid bilayer to mimic the cell membranes, liposomes, as a particulate drug delivery system, are well tolerated by patients with low cytotoxicity [1, 2]. However, the application of liposomes for drug delivery is limited by their short blood circulation time, which consequently has negative influence on the delivery of active ingredients to target locations [3-5]. The mechanism of the rapid clearance is possibly due to the fact that conventional liposomes could easily bind to the serum proteins, thus causing a massive uptake by reticuloendothelial system (RES) [5, 6].

To solve that problem, many approaches were adopted to prolong the blood circulation of liposomes[7]. One of the most common ways is the modification of the liposomal surface by poly(ethylene glycol) (PEG) [8, 9]. Hydrophilic linear PEG chains create a hydrated layer on the surface of liposomes, resulting in decreased adsorption of opsonins and serum proteins thus less recognition and uptake by the RES, which would lead to the extended blood circulation [10].

PEGylated liposomes are generally believed to show no or very low immunogenicity *in vivo*. However, an intravenous administration of PEGylated liposomes in rats, rabbits or Rhesus monkeys could induce rapid clearance of the same liposomes being secondly injected a few days later from the blood stream and extensively increased the accumulation in liver Kupffer cells [11-13], which was named as the "accelerated blood clearance (ABC) phenomenon" [14]. ABC phenomenon would seriously reduce the safety and efficacy of active ingredients. When PEGylated liposomes were used as carrier systems to deliver immunostimulatory factor such as plasmid DNA and oligonucleotides, the repeated injection of PEGylated liposomes would trigger strong immune responses [15]. If the encapsulated drugs possessed a high

cytotoxicity, the occurrence of ABC phenomenon could cause damage to Kupffer cells and result in bacteremia [16].

In addition to the ABC phenomenon, PEGylated liposomes were reported to trigger acute hypersensitivity reactions in sensitive individuals after infusion [17, 18]. These reactions are classified as pseudoallergye and often manifested as flushing and circulatory disturbances [19]. In hypersensitive individuals and animals, the plasma level of SC5b-9 (the terminal complement activation marker of complement system) would be significantly elevated after injections of PEGylated liposomes [20]. Therefore, it was believed that the hypersensitivity reactions caused by PEGylated liposomes strongly correlate with complement activation.

To suppress the ABC phenomenon of PEGylated liposomes, various approaches have been developed and adopted, such as altering the physicochemical properties of liposomes and the administration strategies [21-23]. Studies found that liposomes would not induce the ABC phenomenon after poly(hydroxyethyl L-asparagine) (PHEA) [24] or poly(N-vinyl-2-pyrrolidone) (PVP) modification instead of PEGylation [25]. However, the ABC phenomenon could be still triggered by low dose of PHEA modified liposomes [24]. As for the PVP modified liposomes, its clinic application has been limited, because PVP was found to cause storage disease with the formation of granulomas and interfere with blood coagulation, blood typing and cross matching (see the Federal Register of April 7, 1978 (43 FR 14743), USA). Therefore, seeking an alternative approach remains a great challenge for preparing stealth liposomes without ABC phenomenon and complement activation.

Hyaluronic acid (HA) is a naturally-derived linear glycosaminoglycan composed of the repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid

with β-[1,4] interglycosidic linkage [26]. HA which functions as a supporting material

for organ structural stability and a joint lubricant, exists in living systems with free

and complexed forms [27, 28]. As an endogenous substance, HA is less toxic,

non-immunogenic, biocompatible, and biodegradable [29, 30]. Moreover, HA

modification could present the delivery vehicles with stealthiness in the blood

circulation [29, 31].

In this study, we prepared liposomes modified by HA (HA-Lip) and conducted a

systemic study to address whether the HA-Lip could trigger the ABC phenomenon

and complement activation *in vitro* and *in vivo*.

2. Experimental Section

2.1 Materials and animals

Sodium hyaluronate (HA) with an average molecular weight of 5.6 kDa was

purchased from the Shandong Freda Biopharmaceutical Co., Ltd. (Shandong, China).

Lipoid E80 (purified ovolecithin) and mPEG$_{2000}$-DSPE were obtained from Lipoid

Co., Ltd (Ludwigshafen, Germany).

1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) was obtained from

Corden Pharma Switzerland LLC (Switzerland). Cholesterol (Chol) and

N-hydroxysuccinimide (NHS) was purchased from Kelong Chemical Company

(Chengdu, China). 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride

(EDCI) was purchased from Best Reagent Co., Ltd (Chengdu, China). ELISA kits for

rat plasma thromboxane B2 (TXB2) and human serum SC5b-9 were obtained from

R&D Systems, Inc. (Minneapolis, USA). All the other reagents were of analytical

grade and used without further purification.

Female Wistar rats (220 ± 20 g) and male Vr:CD1 (ICR) mice (20 ± 2 g) were purchased from Dashuo Experimental Animal Co., Ltd (Chengdu, China). All animal experiments were performed in compliance with the guidelines of the Care and Use of Laboratory Animals and approved by the Experiment Animal Administrative Committee of Sichuan University.

2.2 Preparation of liposomes

Common liposomes (cLip) composed of E80:Chol:DPPE at molar ratios of 6:2:1 were prepared by thin-film hydration method as described previously [26] with minor modifications. In brief, the lipids were dissolved in chloroform-methanol (5:1, v/v), evaporated to dryness under reduced pressure in a rotary evaporator and hydrated at 37 °C for 1 h in pH 7.4 phosphate buffer saline (PBS). The obtained multilamellar vesicles were passed through the high pressure jet homogenizer (Nano DeBBE 45, BEE International, Inc., USA) for 10 cycles at 30000 psi. The PEGylated liposomes (PEG-Lip) consisted of E80 : Chol:mPEG$_{2000}$-DSPE with a molar ratio of 6:2:0.4 were also prepared by thin-film hydration method. Briefly, all lipids were dissolved in dichloromethane. Then the thin film was hydrated at 37 °C for 1 h in pH 7.4 PBS after rotary evaporation. Furtherly, the gained multilamellar vesicles were processed by a probe sonicator at 180 W for 3 min. For the preparation of Did-labeled cLip and PEG-Lip, Did dyes were dissolved in the lipid solution before the rotary evaporation, and the other procedures were kept the same.

2.3 HA modification of cLip

The surface modification of liposomes was carried out according to literature [26] with some modifications. Briefly, HA was dissolved in the 100 mM 2-(N-Morpholino)ethanesulfonic acid buffer (Mes buffer) at pH 4.8 and preactivated

by incubation with NHS and EDCI for 1 h at 37 °C. The activated HA was then added into the liposome suspension. The pH was adjusted by 100 mM borate buffer to pH 7.8 and incubated overnight at room temperature. At the end of incubation, HA modified liposomes (HA-Lip) were separated from excess reagents by passing through a Sephadex G75 column using pH 7.4 PBS as eluent.

Did-labeled HA-Lip were prepared with Did labeled cLip accordingly.

2.4 Characterization of liposomes

Particles size distribution and zeta potential were performed on Zetasizer Nano ZS90 instrument (Malvern, UK) by dynamic light scattering (DLS). All measurements of particles size were performed after liposome formulations were diluted by 20 fold in PBS at pH 7.4. The determination of zeta potential for prepared liposomes was carried out without dilution.

The morphology of liposomes was analyzed using a scanning electron microscopy (SEM, Inspect F50, FEI, USA). The liposome formulation was properly diluted and plated on coverslip. After dried naturally, the liposomes were observed by SEM.

The encapsulation efficiency (EE) of liposomes was determined as described below. In brief, the obtained liposomes were passed through a Sephadex G-75 column to remove the free Did. After being disrupted and diluted with methanol, the Did in the liposomes (W_{enc}) was measured by a spectrophotometer (Thermo Scientific Varioskan Flash, Thermo Fisher Scientific, USA) at an excitation wavelength of 644 nm and an emission wavelength of 667 nm. To obtain the total weight of Did (W_{tot}), the same volume of liposomes was directly disrupted with methanol and measured by a spectrophotometer. The EE was calculated by the following formula:

$$EE = W_{enc} / W_{tot} * 100\%.$$

2.5 Pharmacokinetics and distribution of cLip, HA-Lip and PEG-Lip

The pharmacokinetics study of cLip, HA-Lip and PEG-Lip were performed on the female Wistar rats. In brief, Did loaded cLip, HA-Lip and PEG-Lip (150 μg/kg Did) were injected into the rats via tail vein. At the predetermined times (10 min, 30 min, 60 min, 90 min, 120 min, 180 min, 240 min, 360 min, 480 min, 1440 min), blood samples were collected from vena ophthalmica to the heparinized Eppendorf tubes (EP tubes). After centrifuging at 5000 rpm for 5 min, plasma samples were collected into a 96-well black plate and assayed with a spectrophotometer (Thermo Scientific Varioskan Flash, Thermo Fisher Scientific, USA) at an excitation wavelength of 644 nm and an emission wavelength of 667 nm [32].

To study biodistribution, HA-Lip and PEG-Lip, the Did loaded liposomes were intravenously injected into ICR mice. After 4 h, blood was collected and centrifuged at 500 rpm for 5 min to obtain plasma. Then the mice were immediately executed and the intact major organs (including heart, liver, spleen, lung and kidney) were harvested and analyzed using the *in vivo* imaging system (Quick View 3000, Bio-Real, Austria). To calculate the statistical differences, the fluorescence intensity of plasma and organs was assessed by the semi-quantitative analysis of the *ex vivo* fluorescent images.

2.6 Pharmacokinetics and biodistribution of PEG-Lip and HA-Lip after repeated administration

Female Wistar rats were randomly divided into six groups for pharmacokinetics and biodistribution studies. Rats received the first intravenous injection of empty PEG-Lip and HA-Lip at a dose of 0.1 μmoL or 5 μmoL phospholipids/kg. The control

animals were injected with PBS instead of empty liposomes. After seven days, Did loaded PEG-Lip and HA-Lip were administrated intravenously at a dose of 5 μmol phospholipids/kg via tail vein. The injection protocols for PEG-Lip or HA-Lip are presented in Table 1. At predetermined postinjection time points (5 min, 15 min, 30 min, 60 min, 90 min, 120 min and 240 min), blood samples were collected via eye puncture. Mice were sacrificed by cervical dislocation after withdrawing the last blood sample at 240 min, and the livers and spleens were collected. Plasma was obtained and analyzed as mentioned in section 2.5.

The concentrations of Did in tissue samples were also assayed by a spectrofluorometric method. In brief, the tissues were homogenized with two fold of PBS at pH 7.4 (w/v). The homogenates were deproteinized by three fold of acetone and centrifuged at 10,000 rpm for 10 min. The supernatants were collected for analysis.

In addition, the effect of different time intervals on the induction of the ABC phenomenon upon injections of HA-Lip was evaluated. In brief, empty HA-Lip was injected into rats at a dose of 0.1 μmoL phospholipids/kg. After designated intervals (3 days, 7 days and 14 days), the Did loaded HA-Lip was administered again at a dose of 5 μmoL phospholipids/kg. At the predetermined postinjection time points, the blood samples were collected and analyzed as above.

2.7 Determination of serum proteins associated with liposomes

2.7.1 Determination of total serum proteins associated with liposome

Rats were received intravenous injection of empty PEG-Lip (0.1 μmol phospholipids/kg), HA-Lip (0.1 μmol phospholipids/kg) or PBS buffered saline. After

seven days, rats sera were collected and stored at -80 °C until use.

200 μL of liposome suspension (13.5 mM) and 800 μL of rat serum were added into 2 mL EP tubes and incubated for 15 min at a 37 °C environment with 80% final serum concentration. After that, the above mixture was immediately put into ice water for 5 min to end the reaction. The serum was removed by passing through a Sepharose CL-4B column with PBS as eluent.

The total proteins associated with liposome were quantitated by the DC Protein Assay (Bio-Rad laboratories, CA, USA). Bovine serum albumin were used as standard protein and the absorbance was measured at 750 nm using a spectrophotometer (Thermo Scientific Varioskan Flash, Thermo Fisher Scientific, USA). The phospholipid concentration was measured by the colorimetric assay reported by Bartlett et al. [33] . The total lipid concentration was determined according to the molar ratio of the lipid composition. The protein binding index (PBI) was calculated using the following formula:

PBI (g total protein/mol total lipid) = amounts of proteins (g)/molar weight of lipid (mol)

2.7.2 Semi-quantitative determination of IgM in liposome-associated proteins

Plasma samples collected in section 2.7.1 were further subjected to the semi-quantification of IgM absorbed on the liposomes by the Rat IgM ELISA Quantitation Kit (Senxiong Technology Industry Co., Ltd, Shanghai, China) and the absorbance was measured at 490 nm.

2.8 Assay of liposome-mediated human complement activation *in vitro*

Blood was drawn from healthy female volunteers according to the approved local protocols. Blood was allowed to clot at 4 °C, and serum was obtained by centrifugation at 4000 rpm for 10 min and stored at -80 °C for further analysis.

To study liposome-mediated human complement activation *in vitro*, liposomes were added to undiluted serum (liposomes to serum volume ratio of 1:4 with final concentration of 2 mg/mL lipid) in EP tubes and incubated for 30 min at 37 °C. Activation reactions were terminated by adding the "sample diluent" provided with the assay kit. The serum complement activation product SC5b-9 was determined by ELISA.

2.9 Determination of TXB2 level in rat blood

When the complement activation *in vivo* was triggered, the generated anaphylatoxins, such as C3a and C5a, would induce TXA2 releasing from blood cells. TXA2 would be hydrolyzed rapidly to TXB2 within 30 s [34]. Thus the increase of serum TXB2 level could provide evidence for the *in vivo* complement activation. To study liposome-mediated complement activation *in vivo*, the TXB2 level in rat plasma were determined after the injection of liposomes as previously described [35]. Before liposome administration, rat plasma was taken to obtain the baseline parameters. To obtain plasma, blood was collected in EP tubes containing EDTA/0.25 mM indomethacin and then centrifuged at 5000 rpm for 5 min at 4 °C.

Liposome formulations (40 mg/kg body) and PBS (with a same volume) were injected intravenously in rats via tail vein. Blood samples were harvested at 8 min and 60 min post-liposome adimistration to obtain plasma. The plasma TXB2 levels were analyzed by ELISA.

2.10   Statistical analysis

The statistical differences among groups were evaluated using the ANOVA. All data are expressed as mean ± SD (standard deviations). $P < 0.05$ and $P < 0.01$ were considered to be statistically different and statistically significantly different, respectively.

3.   Results

3.1 Characterization of liposomes

As shown in Table 2, the average particle size of cLip was about 120 nm and slightly increased after HA modification. The zeta potential of all obtained liposomes displayed negative charges and HA modification appeared to reduce the zeta potential of cLip. From the images of SEM, it was shown that liposomes displayed uniform diameters in the nanometer scale, which was consistent with the DLS results (Fig. 1). Using Did as the model compound, the encapsulation efficiency (EE) of all liposome formulations under investigation was above 90% (Table 2).

3.2 Pharmacokinetics and distribution of cLip, HA-Lip and PEG-Lip

Nano-carriers with HA modification were demonstrated to show stealthiness in the blood circulation [29, 31]. In addition, HA could specifically bind with CD44 receptors which are over expressed on various tumor cells such as ovarian cancer, colon cancer, and prostate cancer [26, 36]. Thus, HA modified nano-carriers represent a viable strategy to improve the targetability of nanocarriers to tumor cells [29, 37].

To determine the stealthiness of HA-Lip, the pharmacokinetic properties of Did loaded cLip, HA-Lip and PEG-Lip in rats were investigated. As shown in Table 3 and

Fig. 2, the CLz value of HA-Lip (2.67 ± 0.58 mL/h/kg) was decreased significantly (P < 0.05), compared with that of cLip (5.00 ± 1.00 mL/h/kg). With the decrease in the *in vivo* clearance, the AUC_{0-t} value of HA-Lip (9.62 ± 0.32 μg/mL*h) was increased significantly (P < 0.01), compared with that of cLip (4.92 ± 0.57 μg/mL*h). In addition, the terminal half-life $t_{1/2}$ ($t_{1/2}z$) and MRT_{0-t} of HA-Lip was also increased compared to that of cLip (P < 0.05). Our results provided strong evidence for the long circulation properties of HA-Lip in rats, despite that the circulation time of HA-Lip was slightly shorter than that of PEG-Lip.

Furthermore, the biodistribution of cLip, HA-Lip and PEG-Lip in mice was shown in Fig. 3. The liver distribution of cLip was obviously higher than that of HA-Lip and PEG-Lip, which suggested that the stealthiness of HA-Lip in the bloodstream was attributed to less RES uptake in liver.

3.3 Effect of repeated injections on pharmacokinetics and biodistribution of PEG-Lip and HA-Lip

To increase circulation time *in vivo*, PEGylation have been extensively studied and applied in manufacturing nanoscale carriers such as liposomes and nanoparticles [8, 38]. However, after repeated administrations of PEGylated nanoparticles in the same animal, accelerated blood clearance was observed, which is known as the ABC phenomenon [11, 14, 39]. Since HA-Lip demonstrated long circulation profile in blood, whether the repeated injections of the HA-Lip would induce the ABC phenomenon remains unknown.

With repeated injections of PEG-Lip, the plasma concentration of Did was significantly decreased compared with that of the control group (Fig. 4A). The blood clearance of a pre-injection with low dose PEG-Lip (0.1 μmol/kg) was increased

dramatically compared with that of high dose PEG-Lip (5 μmol/kg). The biodistribution in liver also showed an obvious increase ($P < 0.01$) at 4 h after the second dose (Fig. 4B), while the spleen accumulation remained unchanged. As for HA-Lip, no ABC phenomenon was observed after repeated administration with an time interval of 7 days. The accumulations in liver and spleen also remained unchanged compared with the control group (Fig. 5). Moreover, the ABC phenomenon did not occur after repeated injections of HA-Lip at different time intervals (3, 7, 14 days, Fig. 6).

3.4 Correlation between serum proteins associated with liposome and pharmacokinetics of liposome

Serum proteins bonded with liposomes are considered a dominate factor in the accelerated clearance of liposomes, and a higher level of proteins bound was related to a faster clearance of the liposomes [40]. As shown in Fig. 7A, incubated with rat serum that had received a prior injection of PEG-Lip, the PBI value for PEG-Lip were significantly higher than that of PEG-Lip incubated with serum from normal animals ($P < 0.05$). Therefore, the more proteins bound on the secondly injected PEG-Lip led to its faster clearance from blood. As for HA-Lip, the first administration of HA-Lip did not induce the increased association of serum proteins with the secondly injected HA-Lip (Fig. 7A), and as a result, the pharmacokinetics of the second injection of HA-Lip was not changed.

Doxil®, a PEGylated liposomal doxorubicin, was found to trigger the hypersensitivity reactions among sensitive individuals [17], which indicated that PEG-Lip could induce unexpected immune response. IgM emerged as the first

antibody produced in a humoral immune response plays a prominent role in the primary stage of immunity. IgM was believed to stimulate the protein C system, which resulted in augmenting the protein C receptor-mediated endocytosis or phagocytosis of foreign materials [13, 41]. It was shown in Fig. 7B that the amount of IgM bound on the PEG-Lip incubated with the serum from PEG-Lip treated rats was much more than that of PEG-Lip incubated with native rat serum ($P < 0.01$). As a result of IgM activation, the receptor-mediated endocytosis of the secondly injected PEG-Lip by liver macrophages was significantly enhanced. Therefore, its liver uptake was increased, and consequently the blood clearance was greatly accelerated (Fig. 7B). As for HA-Lip, the prior administration of HA-Lip did not induce the increase of the bound IgM on the secondly injected HA-Lip (Fig. 7B).

3.5 Liposome-mediated complement activation *in vitro* and *in vivo*

The PEGylated liposomal doxorubicin (Doxil®) was proven to trigger the complement activation, which would induce the hypersensitivity reactions among sensitive individuals [20, 42]. In this study, the complement activation of PEG-Lip and HA-Lip *in vitro* was investigated using the fresh human serum and the complement activation *in vivo* was performed in rats. For the *in vitro* study, the terminal complement activation pathway markers SC5b-9, a measure of the whole complement cascade activation, was quantified. The results clearly showed that the HA-Lip did not induce the complement activation in the human serum (Fig. 8A). However, with the PEG-Lip, the SC5b-9 levels were much higher than the control group values ($P < 0.01$). Next, the *in vivo* complement activation in rats was examined. After intravenous administration of liposomes, the TXB2 level of rat blood was determined. As shown in Fig. 8B, PEG-Lip administration induced a significant increase in rat plasma TXB2 level on a time scale of minutes and returned to the background level at

60 min, which was consistent with the feature of complement activation [40]. It was reported that the complement activation-related haemodynamic changes would start within 1-2 min after injection of the activators, and the related parameters return to normal after 10-15 min [40]. In contrast to PEG-Lip, intravenous injection of HA-Lip did not alter the rat plasma TXB2 level (Fig. 8B).

4. Discussion

PEGylation is one of common pathway to realize the stealthiness of liposomes in blood circulation, and PEGylated liposomes are generally believed to be a safe carrier with no or very low immunogenicity *in vivo*. However, it was extensively reported that PEGylated liposomes could cause the ABC phenomenon after repeated injections [23, 43, 44]. In addition, PEGylated liposomes could trigger acute hypersensitivity reactions as a result of their complement activation in sensitive individuals [45]. These results illustrate that PEGylation of liposomes could cause potential severe immune response. To prepare alternative stealthy liposomes with reduced immune response, HA was used to modify liposomes in this study.

HA with different molecular weight (5.6 kDa, 250 kDa and 1000 kDa) have been chose to modify the liposomes. However, HA of 1000 kDa in water is easy to gel. The particle size of liposomes modified by HA of 250 kDa would increase to about 200 nm. What's more, the more the molecule weight of HA is, the higher the affinity with CD44 receptor is,[46] and there is over-representation of CD44 receptors on the liver. [47] Therefore, in order to realize the stealth of liposomes in blood circulation, HA with molecule weight of 5.6 kDa was chose to prepare the HA coated liposomes.

Nano-carriers with HA modification were believed to be a viable strategy to improve the targetability of nanocarriers to tumor cells. In our another study,

liposomes modified by HA with molecule weight of 5.6 kDa were used to study in the xenografted melanoma B16f10-burding mice. It was shown that HA modified liposomes represented just a slight increase of tumor distribution compared with that of cLip (data not shown). This result illustrated that the affinity between HA of 5.6 kDa and CD44 receptor was very weak, which was consist with the related report[46]. What's more, the weak affinity of HA (5.6 kDa) to CD44 receptor was also one reason that the distribution of HA-Lip in liver was lower than that of cLip in this study despite the over-representation of CD44 receptors on the liver. In addition, it suggested that high molecular weight HA modification was the better choice to improve tumor targetability of nanocarriers.

The dose of first injection, time interval and physicochemical properties of liposomes (including particle size and zeta potential) have been reported to change the ABC phenomenon. Specifically, the ABC phenomenon could be suppressed with the increase of first administration dose (which was consistent with our results, seen in Fig. 4) [48] and particle size [23]. Regarding zeta potential of liposomes, the positively charged liposomes could induce much weaker ABC phenomenon than the negatively charged liposomes, which is likely due to the shorter blood circulation time of the positively charged liposomes [23]. The ABC phenomenon would be the strongest at 6-7 days after the first injection [14]. In addition to the above impact factors, different species, such as rats, rabbits, rhesus monkeys and mice, were adopted to investigate the ABC phenomenon. Dams et al. [14] found that the ABC phenomenon was demonstrated in rats and rhesus monkeys following repeated injection of PEGylated liposomes (composing of partially hydrogenated egg-phosphatidylcholine (PHEPC), Chol and PEG_{2000}-DSPE), but not in the outbred Swiss mice. Ishida et al. [22] investigated the effect of the physicochemical properties of initially injected

liposomes (composing of HEPC, Chol and PEG$_{2000}$-DSPE) on the ABC phenomenon and their study reported that an obvious ABC phenomenon could be observed in Std:ddY mice. In addition, Tagami et al. [49] also found that the ABC phenomenon could be induced in Std:ddY mice upon repeated injections of PEG-coated cationic liposomes (composed of DC-6-14, POPC, Chol, DOPE and PEG$_{2000}$-DSPE). These results in mice were controversial, which might be due to the differences in the types of mice and phospholipids. However, no systematic study has been conducted on the effect of different mice species and phospholipids on the ABC phenomenon. In this study, we observed the obvious ABC phenomenon of PEGylated liposomes in rats, which was similar to the previous literature [23].

The influence factors on the ABC phenomenon were studied abroad, but the mechanism behind the ABC phenomenon remained largely unknown. Wang et al. [50] showed that the first injection of PEGylated liposomes could promote a strong IgM response against PEG and weak responses against other lipids. The amount of IgM, concomitant complement activation and the magnitude of the ABC phenomenon triggered by those liposomes appeared to be interrelated. These results together suggested that the IgM is secreted in response to the first administration of liposomes and then the IgM binds to the second injected liposomes. The binding subsequently elicits the complement activation, which will result in the enhanced blood clearance and hepatic uptake of foreign materials (the second liposomes) [13]. Our results in this study showed that the prior administration of HA-Lip did not induce the increase of the bound IgM on the secondly injected HA-Lip (Fig. 7B), which can be explained by the fact that HA is an endogenous substance existing in living systems in free and complexed forms and HA molecules from different sources share the same primary structure [51], so HA is biocompatible and does not induce host immune response.

These results were consistent with the above findings and hypothesis. However, Ishihara et al. [25] found that there would be the obvious ABC phenomenon at day 7 after a series of three injections of PEG-Lip with 7 days intervals, but no or minimum IgM response was observed, which implies that the IgM level was not always correlated with the ABC phenomenon extent.

Moghimi et al. [35] found the removal of the negative charges on the phosphate moiety of phospholipid-mPEG by methylation could prevent complement activation of PEGylated liposomes. Thus, the anionic charge on the phosphate moiety of phospholipid-mPEG conjugate was believed to play an important role in complement activation and anaphylatoxin production. However, the complement activation level was significantly higher for PEG-Lip than that of HA-Lip, but the phosphate moiety of phospholipid in HA-Lip was not changed and particle surface charge of HA-Lip also remained negative. Therefore, the phosphate moiety of phospholipid and its anionic charge may not be the major reason for complement activation.

In summary, repeated administrations of PEG-Lip could trigger not only the ABC phenomenon accompanied by a substantially increased accumulation in the rat liver but also the complement activation in human serum *in vitro* and in rat blood *in vivo*. On the contrary, the surface modification of cLip with HA presented a viable strategy to achieve good stealth property in circulation without inducing ABC phenomenon and complement activation after repeated administration. Thus, HA, as an alternative to "PEG" modification, represent an effective and promising delivery strategy, which may greatly increase the utility of liposomes as a targeted delivery vehicle with prolonged circulation *in vivo*.

Acknowledgements

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Table 1 The injection protocols for PEG-Lip and HA-Lip.

Group	First injection (empty liposomes)	Second injection (Did loaded liposones)
I	PBS	PEG-Lip (5 μmol /kg)
II	PBS	HA-Lip (5 μmol /kg)
III	PEG-Lip (0.1μmol /kg)	PEG-Lip (5 μmol /kg)
IV	PEG-Lip (5 μmol /kg)	PEG-Lip (5 μmol /kg)
V	HA-Lip (0.1μmol /kg)	HA-Lip (5 μmol /kg)
VI	HA-Lip (5 μmol /kg)	HA-Lip (5 μmol /kg)

Table 2 Characteristics of cLip, PEG-Lip and HA-Lip. Data are represented as mean ± SD ($n = 3$).

Code	Particle size (nm)	Polydispersity	Zeta potential (mV)	EE (%)
PEG-Lip	118.7 ± 3.8	0.159 ± 0.012	-10.4 ± 2.0	94.5 ± 2.1
cLip	125.6 ± 5.3	0.287 ± 0.041	-5.3 ± 1.4	92.3 ± 1.8
HA-Lip	137.6 ± 6.2	0.228 ± 0.034	-12.5 ± 3.1	93.8 ± 1.4

Table 3 Pharmacokinetic parameters of Did labeled cLip, HA-Lip and PEG-Lip after intravenous

injection in Wistar rats (mean ± SD, $n = 5$).

Parameters	cLip	HA-Lip	PEG-Lip
AUC_{0-t} (µg/mL*h)	4.92 ± 0.57	9.62 ± 0.32**	12.35 ± 1.21**#
$t_{1/2}z$ (h)	6.24 ± 0.26	8.71 ± 0.41**	13.83 ± 2.27**#
MRT_{0-t} (h)	5.60 ± 0.98	7.69 ± 0.33*	9.35 ± 0.74**#
Vz (mL/kg)	50.00 ± 21.79	34.33 ± 5.67	36.50 ± 4.66
CLz (mL/h/kg)	5.00 ± 1.00	2.67 ± 0.58*	1.40 ± 0.54**##

* $P < 0.05$, ** $P < 0.01$, compared with cLip group. # $P < 0.05$, ## $P < 0.01$, compared with

HA-Lip group.

Figure captions

Fig.1 Scanning electron micrographs of cLip (A), HA-Lip (B) and PEG-Lip (C). Scale bars represent 500 nm.

Fig. 2 Mean plasma concentration of Did after intravenous injection of Did labeled cLip, HA-Lip and PEG-Lip in Wistar rats via tail vein at an equivalent dose of 150 μg/kg. ($n = 5$)

Fig. 3 (A) *Ex vivo* imaging of main organs of ICR mice at 4 h post-injection with Did labeled cLip, HA-Lip and PEG-Lip. (B) The statistical graphs of the fluorescence intensity of main organs based on the semi-quantitative analysis of the *ex vivo* fluorescence images of mice obtained at 4 h after intravenous injection of Did labeled liposomes. Data represent mean ± SD ($n = 5$), * $P < 0.05$, ** $P < 0.01$.

Fig. 4 (A) Blood clearance (0 - 4 h) of Did in rats after a second injection of PEG-Lip and the relative concentration represents as the relative ratio with the initial concentration (at 5 min) of group I. (B) Liver and spleen distribution (4 h) of Did in rats after a second injection of PEG-Lip and the relative concentration represents as the relative ratio with the liver and spleen concentration of group I, respectively. Group I, animals accepted the first injection of PBS and the second injection of Did loaded PEG-Lip (5 μmol /kg) after 7 days. Group III, animals accepted the first injection of blank PEG-Lip (0.1 μmol /kg) and the second injection of Did loaded PEG-Lip (5 μmol /kg) after 7 days. Group IV, animals accepted the first injection of blank PEG-Lip (5 μmol /kg) and the second injection of Did loaded PEG-Lip (5 μmol /kg) after 7 days. Data represent mean ± SD ($n = 5$), * $P < 0.05$, ** $P < 0.01$.

Fig. 5 (A) Blood clearance (0 - 4 h) of Did in rats after a second injection of HA-Lip and the relative concentration represents as the relative ratio with the initial concentration (at 5 min) of group II. (B) Liver and spleen distribution (4 h) of Did in rats after a second injection of HA-Lip and the relative concentration represents as the relative ratio with the liver and spleen concentration of group II, respectively. Group II, animals accepted the first injection of PBS and

the second injection of Did loaded HA-Lip (5 μmol /kg) after 7 days. Group V, animals accepted the first injection of blank HA-Lip (0.1 μmol /kg) and the second injection of Did loaded HA-Lip (5 μmol /kg) after 7 days. Group VI, animals accepted the first injection of blank HA-Lip (5 μmol /kg) and the second injection of Did loaded HA-Lip (5 μmol /kg) after 7 days. Data represent mean ± SD ($n = 5$).

Fig. 6 Blood clearance (0 - 4 h) of Did in rats after a second injection of HA-Lip. Rats were pretreated with HA-Lip at a dose of 0.1μmol /kg and then the second injection of HA-Lip (5 μmol /kg) were given. The time intervals of two injections were 3 days, 7 days and 14 days. The relative concentration represents as the relative ratio of initial concentration of "7 days" group. Data represent mean ± SD ($n = 5$).

Fig. 7 (A) Amount of total serum protein associated with liposomes. The serum collected from rats at day 7 post-first injection of PBS or liposomes. Each value represents the mean ± S.D. ($n = 5$). ** $P < 0.01$. (B) Semi-quantitative determination of IgM in liposome-associated proteins. The proteins associating with liposomes were prepared by incubation with serum collected at day 7 post-first injection of PBS or liposomes. Each value represents the mean ± S.D. ($n = 5$). ** $P < 0.01$.

Fig. 8 Complement activation by PEG-Lip and HA-Lip. (A) SC5b-9 levels in human serum 30 min after liposome treatment (2 mg lipid/mL) at 37 °C ($n = 5$). Data represent mean ± SD, ** $P < 0.01$, compared with PBS group. (B) Plasma TXB2 levels in rats following intravenous injection of PEG-Lip and HA-Lip at a dose of 40 mg/kg ($n = 5$). Data represent mean ± SD, * $P < 0.05$, compared with 0 min.

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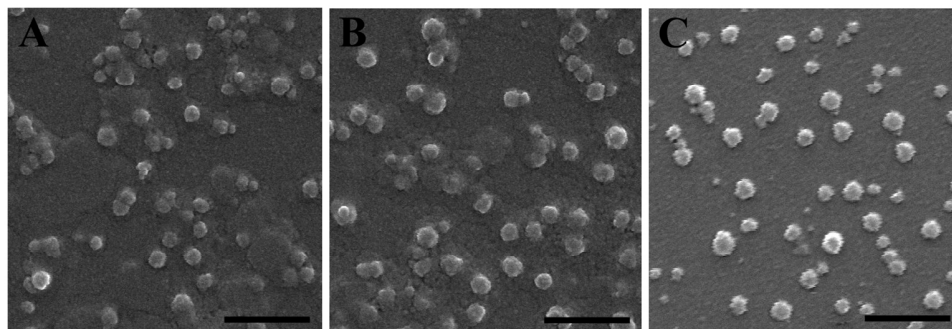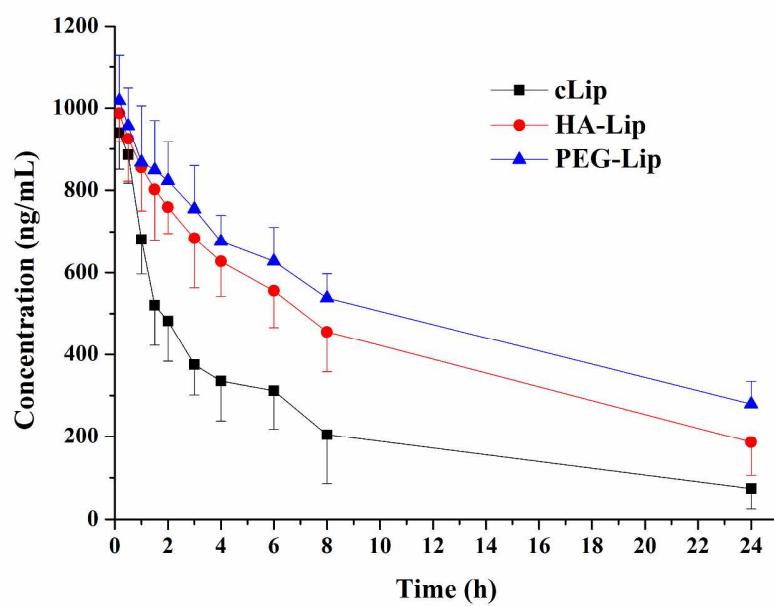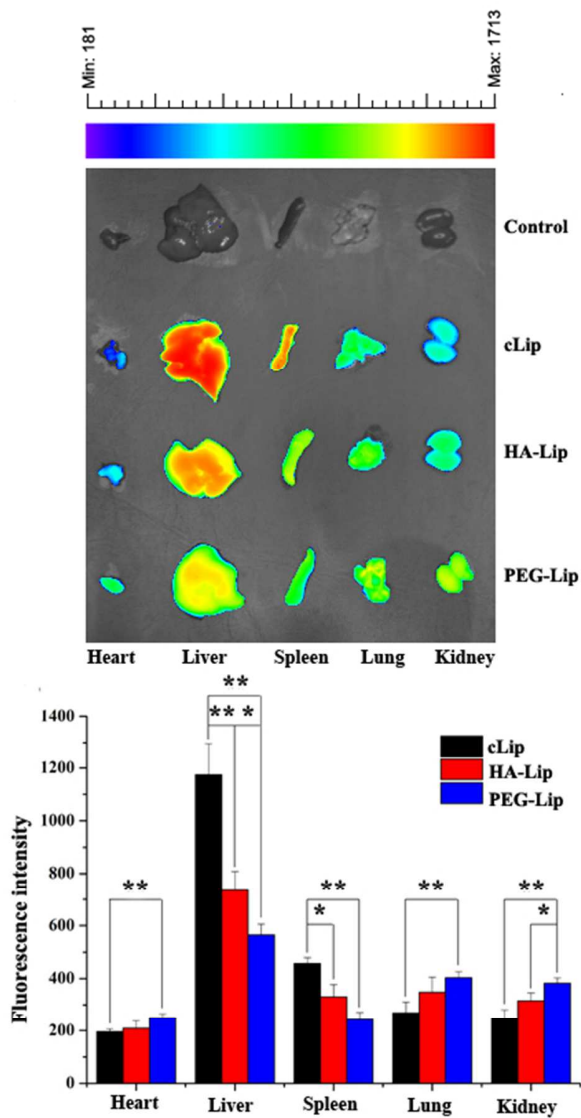53
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Fig.1
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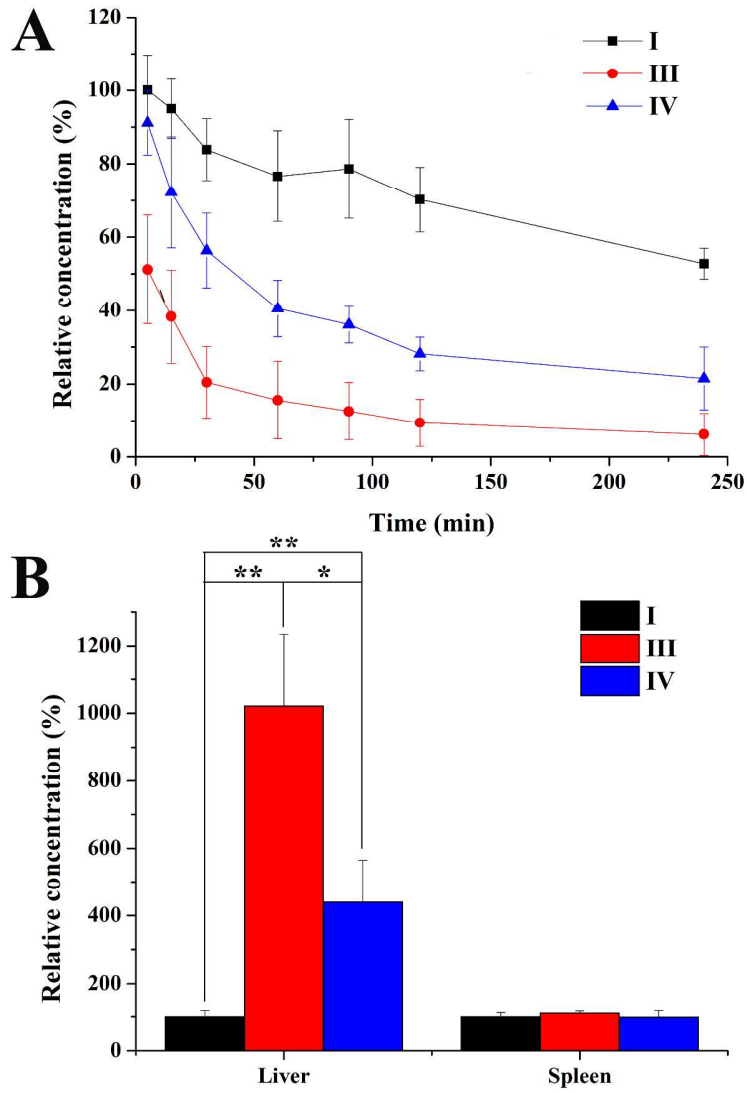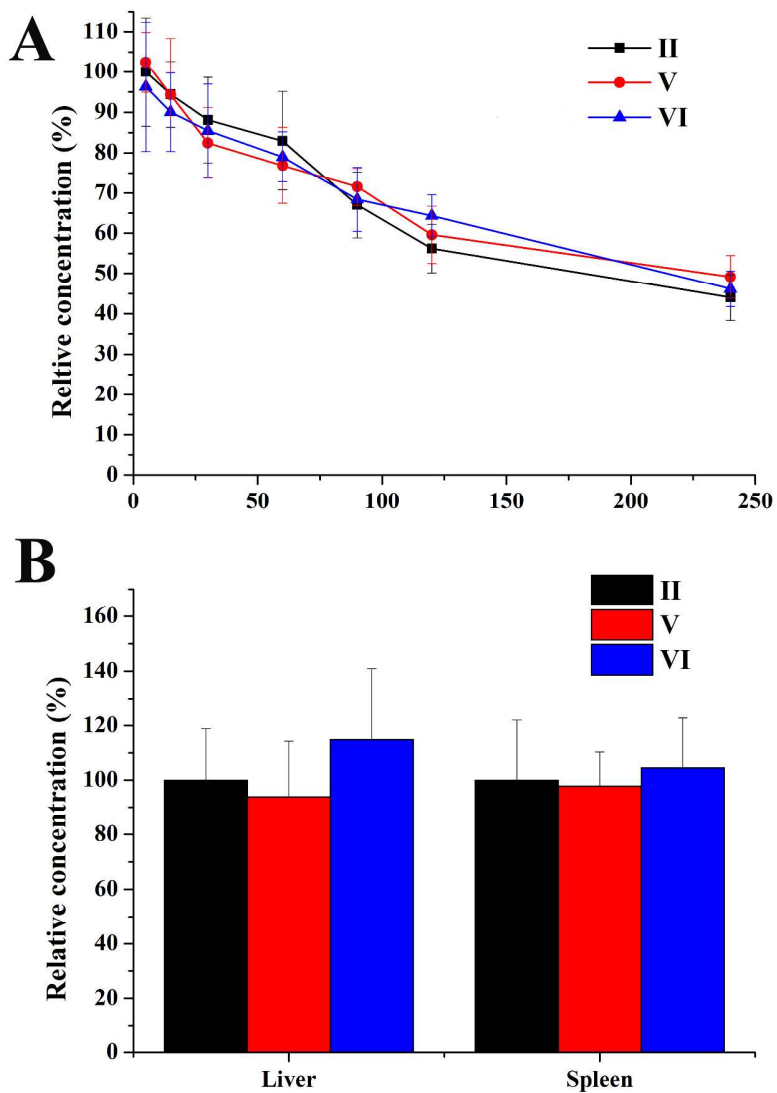
Fig.2
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Fig.3
179x299mm (72 x 72 DPI)

Fig.4
1237x1666mm (72 x 72 DPI)

Fig.5
1237x1583mm (72 x 72 DPI)
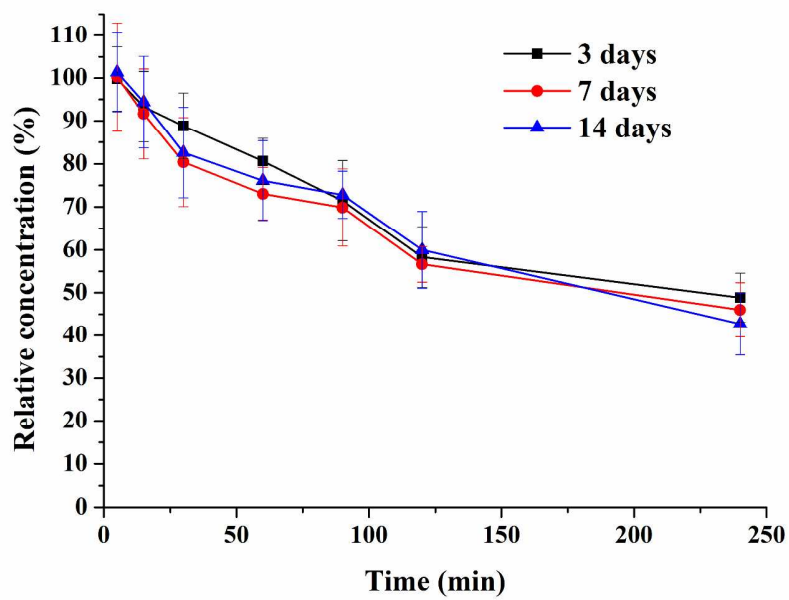
Fig.6
1237x874mm (72 x 72 DPI)

Fig. 7
1237x1666mm (72 x 72 DPI)

HA

EDCI/NHS

cLip

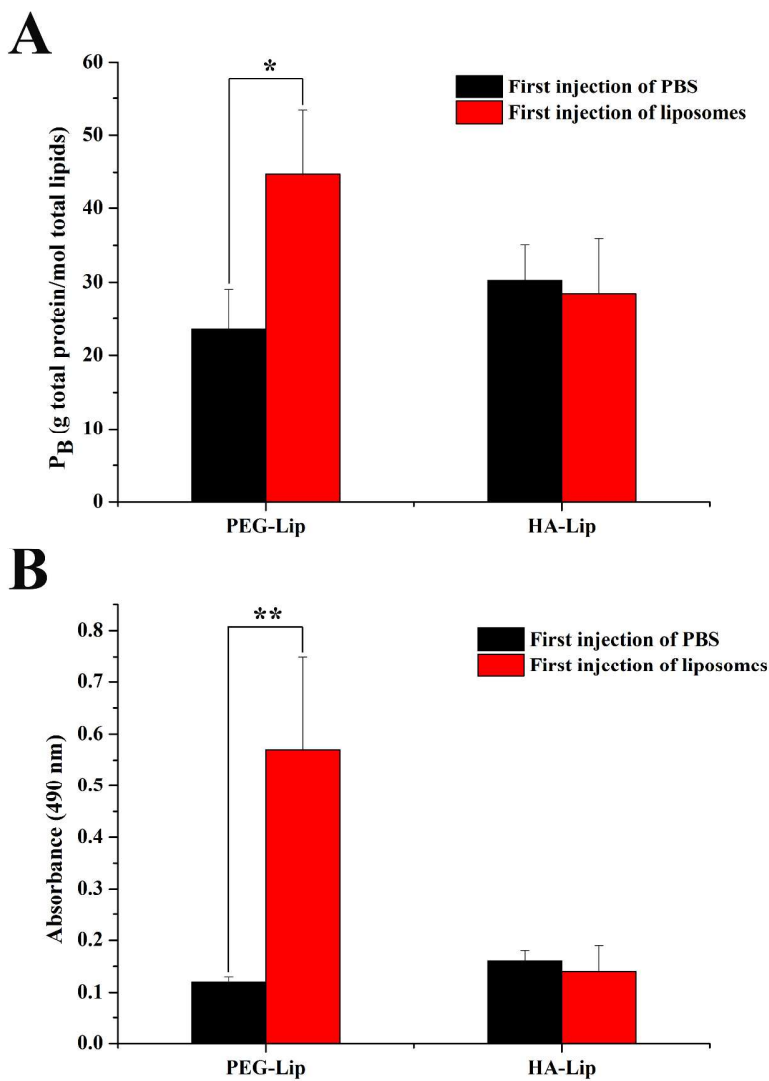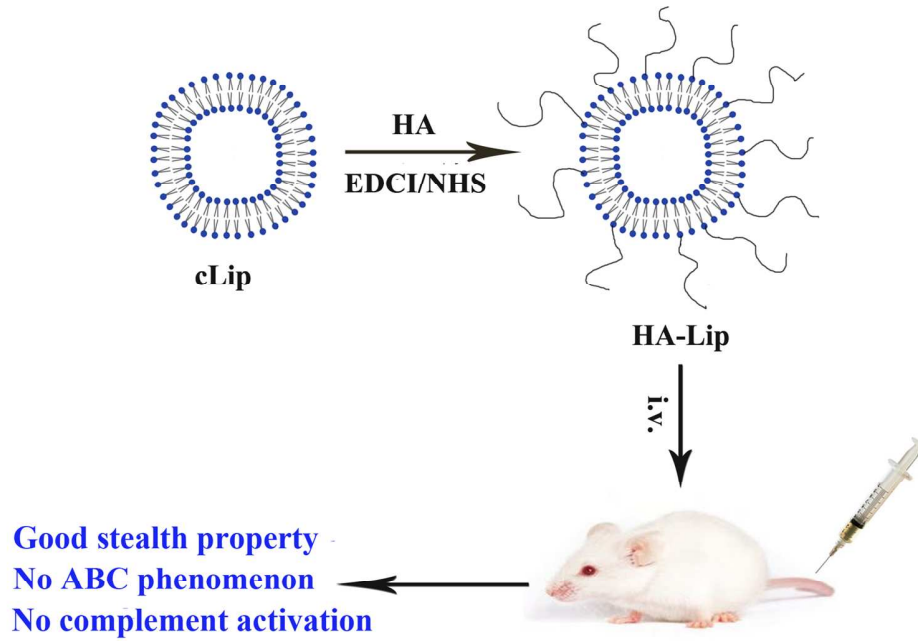**HA-Lip**

i.v.

Good stealth property
No ABC phenomenon
No complement activation

Hyaluronic acid (HA) could be an alternative to "PEG" modification. The HA coated liposomes showed good stealth property in blood circulation and they didn't trigger the complement activation in vitro and in vivo. What's more, there was no ABC phenomenon after repeated administration of HA coated liposomes.
635x453mm (72 x 72 DPI)