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Increase of the pharmacological and pharmacokinetic efficacy of negatively charged polypeptide recombinant hirudin in rats via parenteral route by association with cationic liposomes

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ABSTRACT

Two biodegradable cationic lipids, stearylamine and DC-Chol, were chosen to investigate the effect of cationic lipids on the in vitro and in vivo characteristics of hydrophilic proteins or peptides of low isoelectric point. Thrombin inhibitor recombinant hirudin variant-2 (rHV2) was selected as the model drug. The cationic lipids were found to achieve higher entrapment efficiency of rHV2 in liposomes than zwitterionic lipids. The positively charged liposomes became less positive and relatively stable in serum after loading rHV2. The cationic liposomes induced sustained release of rHV2 in the presence of plasma, significantly prolonged the antithrombotic efficacy and plasma level of rHV2 after intravenous injection in rats in comparison with neutral lipid liposomes, especially for stearylamine group. Both clotting times correlated well with plasma rHV2 levels. No serious adverse events were observed and physical state of rats was satisfactory for all the formulations. Electrostatic interaction between negative charge of rHV2 and cationic liposomes was confirmed and it might affect all the characteristics of rHV2 loaded cationic vehicles. The findings suggest that cationic liposomes may be a potential sustained-release delivery system for parenteral administration of hydrophilic proteins or peptides with low isoelectric point to prolong efficacy and improve bioavailability.

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1. Introduction

The use of peptides or proteins as therapeutic agents becomes increasingly important as more and more such biopharmaceuticals can be produced by genetic engineering. However, it is well known that peptide or protein drugs usually possess very short biological half-life and extreme instability because of proteolytic inactivation and/or degradation. Consequently, a great number of peptides or proteins were incorporated with some kinds of delivery systems, especially liposomes, to obtain beneficial clinical outcomes, including extended circulation time [1,2], increased stability [3,4] and improved efficacy [5,6]. Since most of peptides or proteins are hydrophilic, it is a big challenge to achieve satisfactory entrapment efficiency (EE) in liposomes by the commonly used zwitterionic lipids. As a matter of fact, the EE of liposomal proteins prepared by such lipids is usually less than 35% [7].

Cationic liposomes were usually applied to enhance the incorporation efficiency and cell uptake of gene drugs, including DNA, antisense oligodeoxynucleotide and siRNA [8–11]. The antivascular and antitumor properties of cationic liposomes were also utilized for improving neovascular targeting chemotherapy of anticancer drugs, such as paclitaxel [12–14] and doxorubicin [15]. Recently, a variety of cationic lipid formulations have been demonstrated to enhance the EE of hydrophilic peptides or proteins with low isoelectric point (pI) [1,16–20]. Ken et al. [1] found that the positively charged stearylamine (SA) liposomes entrapped more insulin (pI ≈ 5.3) than zwitterionic or negatively charged liposomes. In another study, superoxide dismutase (SOD) (pI ≈ 4.9) was successfully incorporated into SA liposomes with high EE [16]. On the other hand, the negatively charged lipids were also proved to increase the EE of peptides with high pI [21].

Due to the electrostatic association with cationic lipids, peptides or proteins loaded usually released slowly from the liposomes, and a prolonged plasma level was achieved. Corvo et al. [16] demonstrated that the terminal half-life of SOD increased to 5–10 folds after associated by SA liposomes compared with the free drug. Also, the positively charged liposomes are generally more stable than other kinds of liposomes [22].

One of the major concerns for cationic lipids is the in vivo toxicity. However, it has been reported that the toxicity of intravenous cationic lipid complexes is often dose-dependent [23,24], which means that there will be no serious side effects if the dosing is rationally designed. Secondly, lipids like SA and DC-Chol containing biodegradable linker

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bonds, ester or amide, are relatively less toxic than non-degradable and tertiary amines [23,25]. As a matter of fact, quite a lot of DC-Chol/gene complexes have been approved by FDA for clinical studies [26–28]. Up to now, however, few of the studies related to peptide or protein loaded cationic liposomes have given sufficient details to properly characterize these vehicles, especially for serum stability, in vitro release of drug loaded in the absence or presence of plasma, pharmacodynamic and pharmacokinetic behavior.

In this study, two biodegradable cationic lipids, SA and DC-Chol, were used and a peptide, recombinant hirudin, was selected as the model drug. Hirudin is the most potent thrombin inhibitor [29] and composed of 65-amino acid residues with a molecular mass of approximately 7000 Da and a low pI (pI≈4.0) [29]. However, the therapeutic application of hirudin has been greatly hindered due to its rapid clearance from blood (half-life≈1 h [29]). Several authors have developed sustained-release delivery systems for hirudin, such as PEG–hirudin [30], zinc–hirudin suspension [31], dextran coupled hirudin [32], hirudin–dextran coupled liposomes [33] and hydrogel based hirudin [34], but most of these studies are preliminary. Apparently, it is beneficial to develop a sustained-release delivery system for intravenous (i.v.) administration of hirudin to prolong the action time and minimize the number of injections.

The aim of present study was to explore and evaluate the effect of cationic lipids on the characteristics of rHV2 loaded liposomes in vitro and vivo, including the EE, stability in serum, in vitro release with or without plasma, pharmacodynamics, pharmacokinetics, toxicity as well as the correlation between pharmacological and pharmacokinetic results after i.v. injection in rats.

2. Materials and methods

2.1. Materials

Recombinant hirudin variant-2 (rHV2, 5000 ATU/mg determined by chromogenic thrombin assay, College of Life Science, Peking University, Beijing, China) was obtained by polymerase chain reaction-directed mutagenesis through expressing in *Escherichia coli*. Egg phosphatidylcholine (EPC) was purchased from Lipoid GmbH (Germany). DC-Chol (3β-[*N*-(*N*′, *N*′-dimethylaminoethane)-carbamoyl]-cholesterol) was synthesized as previously reported [35] and identified by melting range, ^{1}H-NMR and EI Mass Spectrum, etc. Stearylamine (SA) was obtained from Changzhou Xinhua Active Material Institute (Jiangsu, China). Cholesterol (CH), Triton X-100, Tris-base and thrombin were obtained from Sigma-Aldrich (St. Louis, MO). Both fetal bovine serum (FBS) and DMEM were the GIBCO product of Invitrogen Corporation. Chromozym TH was from Roche Diagnostics (Mannheim, Germany). Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany). Kits for determining activated partial thromboplastin time (APTT) and thrombin time (TT) were purchased from Sun Biotech Co Ltd. (Shanghai, China). Sephadex G-200 was the product of Pharmacia. All other reagents were of analytical grade.

2.2. Preparation of rHV2-loaded liposomes

2.2.1. Preparation of rHV2-loaded sonicated unilamellar vesicles (rHV2-SUVs)

The rHV2-SUVs were prepared by film-dispersion method [36]. EPC and CH were mixed at a 2:1 molar ratio (15 μmol total lipids) in chloroform and dried under reduced pressure by rotary evaporation at 25 °C. The obtained lipid film was then hydrated in 2 ml of distilled deionized water (ddH_2O) containing rHV2 (0.2 mg/ml). The sample was vortexed for 2 min and then sonicated for 10 min in an ultrasonic bath at 25 °C, thus the neutrally charged liposomes, i.e. rHV2-EPC-SUVs, were obtained. Cationic lipid (CL), SA or DC-Chol, was added to lipid mixture at a molar ratio of EPC/CH/CL=2:1:0.5 to get two kinds of rHV2-CL-SUVs, respectively.

2.2.2. Preparation of rHV2-loaded reverse-phase evaporation vesicles (rHV2-REVs)

The rHV2-REVs were prepared as described by Szoka [37]. Briefly, the lipid mixture as used in SUVs preparation was completely dissolved in 5 ml of chloroform. Aliquot 1 ml of ddH_2O containing rHV2 (0.4 mg/ml) was added. The two-phase system was sonicated for 40 s in a needle-type sonicator (120 Hz) at 0–5 °C to get a stable w/o emulsion. Chloroform was then removed under vacuum in a rotary evaporator at 25–30 °C until a gel phase appeared. At this point 1 ml of drug-free ddH_2O was added and subsequent evaporation led to the inversion of gel to liquid. The optimization of rHV2-REVs was based on the EE results of rHV2 in different formulations by varying CL ratio and aqueous phase, including ddH_2O, 5% glucose (wt.%), PBS and 1/10 PBS.

2.3. Interaction between lipids and rHV2

Three types of blank SUVs were used: EPC-SUVs, SA-SUVs and DC-Chol-SUVs. The total lipid content of blank SUVs was maintained at 5.0 mg/ml. Then equal volume of ddH_2O containing rHV2 (0.4 mg/ml) was added respectively. Before and after incubation at room temperature for 2 h, the particle size and zeta potential were measured. The amount of associated rHV2 was obtained by passing the mixture down through Sephadex G-200 and then determined by HPLC. All measurements were performed in triplicate.

2.4. Characteristics of rHV2-REVs

2.4.1. Determination of EE

To determine the EE of rHV2-loaded liposomes, the bound drug was obtained by Sephadex G-200 (1×15 cm) as described above. The EE was the ratio of liposomal amount of rHV2 to the total amount of rHV2 in the liposomal suspension disrupted by 5% Triton X-100.

The rHV2 concentration was determined by HPLC (HP 1100, USA) with Kromasil C18 column (250×4.6 mm, 5 μm, Akzo Nobel, Sweden). The mobile phase was a mixture of NaH_2PO_4 (0.10 M), Na_2SO_4 (0.05 M) and acetonitrile at a ratio of 37:37:26 (v/v/v) with pH of 3.8–4.0. Flow rate was 1.0 ml/min with detection at 214 nm. Aliquots 20 μl of samples were injected into HPLC system. The peak area of rHV2 was recorded and the concentration was calculated from standard curve.

2.4.2. Determination of zeta potential of blank REVs and rHV2-REVs

Zeta potential of blank REVs and rHV2-REVs was measured by Malvern Zetasizer (ZEN 3600, Malvern Instruments, England). All samples were diluted to total lipid content of 0.5 mg/ml. The measurements were performed in triplicate.

2.4.3. Stability of blank REVs and rHV2-REVs in the presence of serum

Blank REVs or rHV2-REVs (100 μl) were incubated with 1 ml serum (10% FBS in DMEM) at 37 °C. Aliquot 50 μl of the mixture diluted by 5% glucose (800 μl) was used to determine absorbance at 630 nm to quantify turbidity changes as time increased.

2.4.4. In vitro release of rHV2 from REVs

Release of rHV2 from various REVs was performed against 5% glucose using the dialysis bag (Green Bird Science & Technology Development, China) with molecular weight cut-off of 50 kDa. Aliquots 0.5 ml of rHV2-REVs diluted at 1:1 with 5% glucose or plasma were placed in a dialysis bag immersed in 10 ml of 5% glucose and then incubated in thermostatic shaker (100 rpm, 37 °C). At specific times, aliquots 50 μl of the receiver medium were taken and replaced with 50 μl fresh medium. After 48 h, the suspension was disrupted by 5% Triton X-100. The amount of rHV2 in the samples was analyzed by HPLC and the cumulative release percentages of rHV2 were calculated.

2.4.5. Pharmacodynamic and pharmacokinetic studies of rHV2-REVs

2.4.5.1. Animals. For each optimized rHV2 formulation studied, male Sprague–Dawley (SD) rats (Vital Laboratory Animal Center, Beijing, China), weighing about 250 g were acclimated for one week under standardized environment with free access to standard food and water. All studies were conducted in accordance with the approval of Institutional Authority for Laboratory Animal Care.

2.4.5.2. Pharmacodynamic experiments. Four groups of SD rats (five to six rats per group) were used to determine the pharmacological efficacy of rHV2 formulations as follows: (1) rHV2 in 5% glucose, (2) rHV2-EPC-REVs, (3) rHV2-SA-REVs, (4) rHV2-DC-Chol-REVs. All rHV2 formulations (containing 0.2 mg/ml rHV2) were given intravenously to rats by tail vein, at the doses of 0.5 mg/kg.

Blood samples (about 0.5 ml) for evaluation of clotting times were collected under anesthesia from the orbital sinus before and at specific time points after administration. Blood samples were placed in tubes containing 1.75:10 (v/v) 3.8% sodium citrate and centrifuged at 5000 rpm for 10 min. The plasma obtained was frozen at −20 °C until assay. The APTT and TT for each sample were determined by commercially available kits following corresponding instructions. Anticoagulant effect of rHV2 for different groups was expressed as prolongation in APTT and TT compared with normal values before injection for each rat.

2.4.5.3. Pharmacokinetic experiments. The grouping, dosing and blood-taking procedures were the same as above. Plasma concentration of rHV2 was determined by the chromogenic thrombin substrate assay just as described in previous report [38]. The plasma pharmacokinetic parameters were assessed by WinNonLin (Pharsight, Mountain View, CA, USA), using non-compartmental analysis. The initial plasma rHV2 concentration (C_0), terminal rate constant (k_e), terminal half-life ($t_{1/2}$), area under the plasma concentration-time curve up to last time (AUC_{0-t}) or infinite time (AUC) and mean residence time up to last time (MRT_{0-t}) for each rat were obtained.

During 48 h after receiving rHV2 formulations, animals were on observation for any serious side effects, acute toxicity or death.

2.5. Statistical analysis

All data are shown as means±standard deviation (S.D.) unless particularly outlined. Student's unpaired *t*-test and one-way analysis of variance (ANOVA) were performed in statistical evaluation. The significance level was set to be $p<0.05$.

3. Results and discussion

3.1. Effect of CL on rHV2 entrapment in SUVs and REVs

Table 1 shows that only 8.4% of rHV2 could be encapsulated in EPC-SUVs, which might be due to limited aqueous interior for SUVs; however, approximately two-fold more rHV2 was entrapped by EPC-REVs with the same lipid composition, 2:1:0.5 (EPC/CH/CL, molar ratio). This result confirms previous finding of REVs encapsulating more hydrophilic drugs [37]. It was then proved that the addition of 15 mol% CL significantly enhanced the EE of rHV2 ($p<0.05$), irrespective of the structure of CL or preparation method. These data reveal that enhanced entrapment of rHV2 can be achieved by using cationic lipid. Moreover, the two positively charged lipids showed a different behavior for different liposome type. The DC-Chol-SUVs entrapped more rHV2 than SA-SUVs (43.1% vs. 19.7%), while DC-Chol-REVs loaded less rHV2 than SA-REVs (59.0% vs. 66.7%).

3.2. Optimization of rHV2-REVs

The rHV2-REVs were selected for formulation optimization because of its preferable encapsulation ability (Table 1). As was shown in

Table 1
Effect of preparation method, CL content and aqueous phase on entrapment efficiency of rHV2 liposomes (mean±S.D., $n=3$)

Liposome type	EPC/CH/CL (molar ratio)	Aqueous phase	Entrapment efficiency (%)		
			EPC group	SA group	DC-Chol group
SUVs	2:1:0.5	ddH$_2$O	8.4±2.7	19.7±3.9	43.1±6.7
REVs	2:1:0.25	ddH$_2$O	N.A.[a]	41.5±3.1	32.7±5.0
	2:1:0.5	ddH$_2$O	23.0±3.2	66.7±5.6	59.0±2.5
	2:1:1	ddH$_2$O	N.A.	74.6±3.4	61.1±3.5
	2:1:0.5	5% glucose	20.5±4.7	60.6±5.1	63.4±8.5
	2:1:0.5	1/10 PBS[c]	10.3±2.2	19.2±3.2	20.4±1.6
	2:1:0.5	PBS[b]	4.8±3.2	8.0±1.9	9.6±4.5

[a] Not applied.
[b] The PBS solution contains 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4 and 1.4 mM KH_2PO_4.
[c] The 1/10 PBS represents 10-fold dilution of PBS with ddH$_2$O.

Table 1, the entrapment of rHV2 in cationic REVs increased with CL content. When CL proportion increased from 2:1:0.25 to 2:1:0.5, the percentage encapsulation of rHV2 increased almost by 100% and 50% for DC-Chol and SA group, respectively. However, further increment resulted in a disproportional rise in rHV2 entrapment. This nonlinear effect revealed a saturated tendency of rHV2 entrapment for high CL ratio. Similar results were obtained for SOD cationic liposomes [16].

The data indicate that the aqueous phase for rHV2 dissolution also plays an important role in the encapsulation. With the lipid composition of EPC/CH/CL=2:1:0.5 (molar ratio), more than 55% of rHV2 was incorporated for cationic groups when rHV2 was dissolved in ddH$_2$O or 5% glucose. While dissolved in 1/10 PBS or PBS, less than 22% or 10% of rHV2 was entrapped, respectively. This phenomenon is probably caused by the Na^+ and Cl^- ions preventing the electrostatic interaction between rHV2 and CL, since a non-solute, glucose, had less effect on the entrapment. Similar findings were observed by previous studies [10,37].

Finally, the composition of EPC/CH/CL=2:1:0.5 and 5% glucose were selected to prepare rHV2-REVs for further characteristic studies.

3.3. Interaction between SUVs and rHV2

Fig. 1a shows that blank SA-SUVs and DC-Chol-SUVs exhibited particle size of 125.0 and 95.9 nm, respectively. After addition of rHV2, the values increased to 151.7 and 128.6 nm respectively. Contrarily, the value was not affected for EPC group. These phenomena are consistent with previous reports for polynucleotides cationic liposomes [39,40]. In addition, zeta potential was significantly reduced for both cationic groups by addition of rHV2 ($p<0.05$) (Fig. 1b), from +52.1 mV down to +40.2 mV for SA group, and from +52.7 mV down to +27.6 mV for DC-Chol group. Only a little rHV2 (2.1%) was incorporated with EPC-SUVs, whereas 18.3% and 26.9% of rHV2 was associated with SA-SUVs and DC-Chol-SUVs, respectively (Fig. 1c). All the changes demonstrate the existence of interaction between negatively charged rHV2 and positively charged SA or DC-Chol. The interaction is presumably the reason for high entrapment of rHV2 in cationic liposomes.

3.4. Zeta potential changes of REVs after loading rHV2

The rHV2 loading made great changes in the zeta potential of blank cationic liposomes, from +39.9 mV down to +27.1 mV for SA group, and from +38.8 mV down to +26.8 mV for DC-Chol group. It is obvious that the positive charges are partly neutralized by rHV2. There was no significant difference between two cationic groups, which suggests that the effect of rHV2 on the two kinds of cationic liposomes is almost similar, independent on the structure of CL. On the contrary, a minor change of zeta potential was observed for EPC-REVs when rHV2 was encapsulated, from −24.6 mV to −20.0 mV.

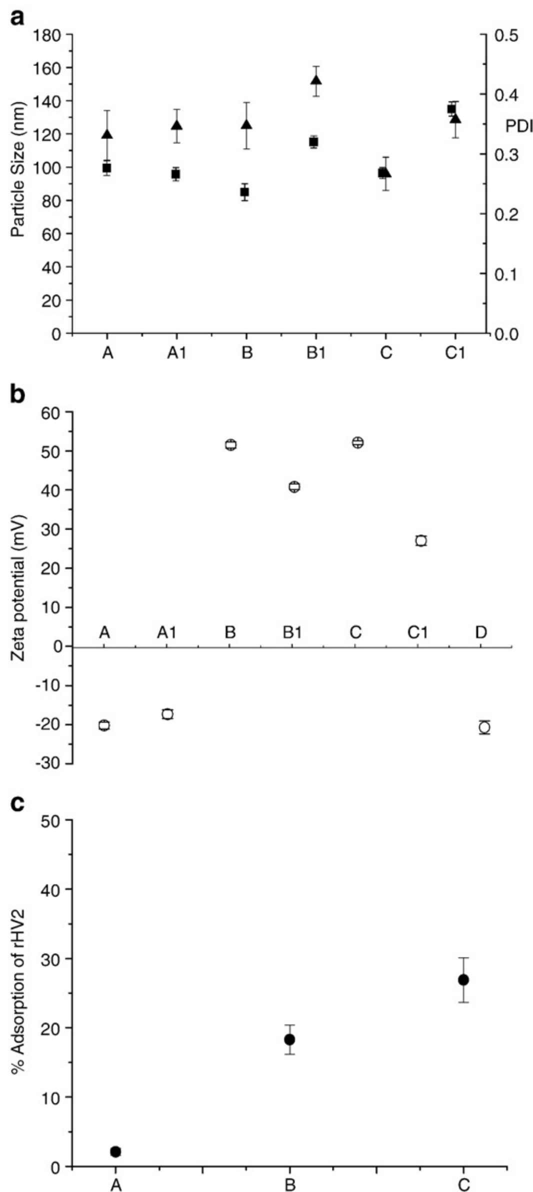

Fig. 1. Summary of interaction between SUVs and rHV2. (a) Particle size (▲) and PDI (■) change; (b) Zeta potential change; (c) Percentage of rHV2 adsorbed by SUVs. (A) blank EPC-SUVs; (A1) EPC-SUVs incubated with rHV2; (B) blank SA-SUVs; (B1) SA-SUVs incubated with rHV2; (C) blank DC-Chol-SUVs; (C1) DC-Chol-SUVs incubated with rHV2. (D) rHV2 solution. The concentration of rHV2 and total lipids in the samples was 0.2 and 5.0 mg/ml, respectively. Data are expressed as means±S.D. (n=3).

3.5. Serum stability of rHV2-REVs

Fig. 2 shows the effect of loading rHV2 on the serum stability of REVs. The medium (5% glucose) itself could not change the absorbance value of serum at 630 nm. No obvious increase in absorbance occurred for EPC group, suggesting that no aggregate appeared in the solution. The turbidity of serum increased rapidly as soon as incubated with blank cationic liposomes ($p<0.01$ vs. EPC group) and the reason might be the interaction between CL and the proteins in serum. Nevertheless, both the increasing rate and final value of absorbance were lower for rHV2-CL-REVs ($p<0.01$), revealing that the interaction might be partially prevented. The improved serum stability is clearly due to the fact that rHV2-CL-REVs become less positive than CL-REVs (see results in Section 3.4), which is supported by the published results that cationic liposomes with less excess positive charges are helpful for improving their stability in vivo [10]. Additionally, the decrease of turbidity as time increased is probably induced by precipitation of large particles [11].

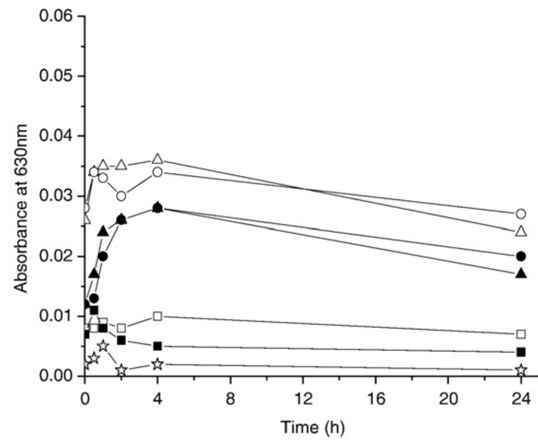

Fig. 2. Serum stability of blank REVs (empty markers) and rHV2-REVs (full markers) for EPC group (■), SA group (▲) and DC-Chol group (●), respectively, using 5% glucose (☆) as control group. Data are expressed as means (n=3).

3.6. In vitro release of rHV2-REVs

The rHV2 released from REVs was presented as a cumulative release percentage of rHV2 to the total amount of rHV2 in liposomes (Fig. 3). Around 70% of loaded rHV2 released from EPC-REVs in 5% glucose after 48 h, whereas significantly less drug released from CL-REVs ($p<0.01$), with cumulative release percentage of 36.15% and

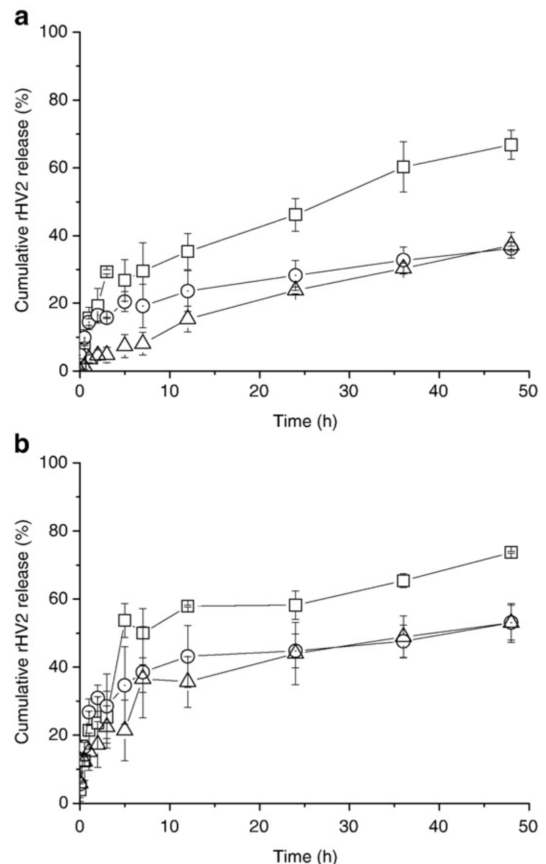

Fig. 3. In vitro release of rHV2 from EPC-REVs (□), DC-Chol-REVs (○) and SA-REVs (△) in 5% glucose (a) or plasma (b) at 37 °C. Data are shown as means±S.D. (n=3).

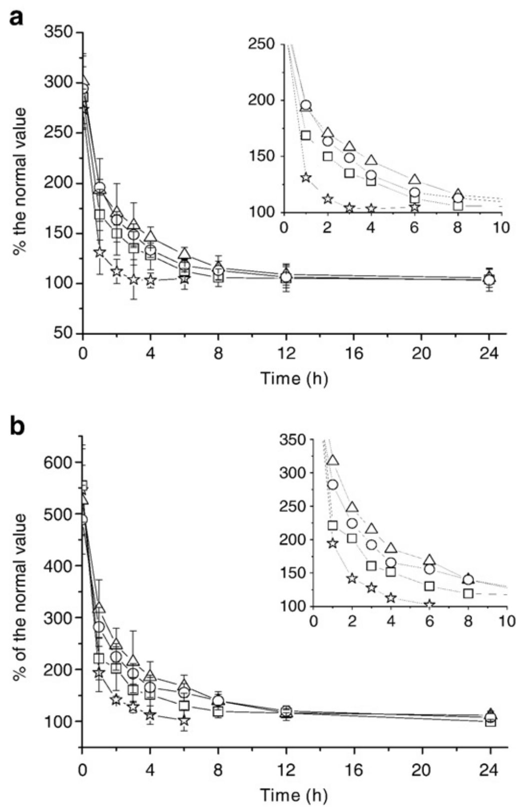

Fig. 4. Profiles of APTT (a) and TT (b) prolongation versus time after i.v. injection of rHV2 solution (☆), rHV2-EPC-REVs (□), rHV2-DC-Chol-REVs (○) and rHV2-SA-REVs (△) in normal rats. Each value is expressed as mean±S.D. (n=5–6). The enlarged parts without error bars on the right top of each figure are used for clearer illustration.

37.12% for DC-Chol-REVs and SA-REVs after 48 h, respectively (Fig. 3a). Fig. 3b shows that both release rate and total drug released for all groups greatly increased in the presence of plasma. In an early time, a large amount of rHV2 released from all formulations tested, with more than 50%, 40% and 35% of total rHV2 released from EPC, DC-Chol and SA liposomes after 10 h ($p<0.01$ between CL and EPC group), respectively. After initial burst, the rHV2 released steadily, and the maximal release percentage was 73.78%, 53.12% and 52.59% for the three REVs after 48 h ($p<0.01$), respectively. These findings indicate that both cationic REVs exhibit sustained-release property for rHV2.

It is clear that there were obvious differences between EPC-REVs and CL-REVs in release rate and total rHV2 release, no matter in the 5% glucose or plasma, again due to the difference in the interaction between rHV2 and different REVs. Although cationic liposomes were more unstable in plasma than EPC-REVs because of aggregation when exposed to the proteins in the plasma [1], the rHV2 release from CL-REVs was still much slower than that from EPC-REVs, suggesting the importance role of the interaction in the release.

Table 2
Pharmacokinetic parameters of rHV2 after i.v. injection of rHV2 formulations in five to six rats at the doses of 0.5 mg/kg (mean±S.D.)

Parameter	rHV2 solution	rHV2-EPC-REVs	rHV2-DC-Chol-REVs	rHV2-SA-REVs
C_0 (ng/ml)	606.98±47.64	577.90±40.04	601.97±27.70	552.50±21.79
k_e (h^{-1})	1.03±0.24	0.33±0.03	0.20±0.03	0.11±0.02*
$t_{1/2}$ (h)	0.71±0.19	2.11±0.19	3.52±0.48	6.47±1.32*
AUC_{0-t} (μg×h/ml)	0.34±0.02	0.77±0.07	1.49±0.35	3.16±0.41*
AUC (μg×h/ml)	0.36±0.03	0.79±0.07	1.53±0.39	3.51±0.51*
MRT_{0-t} (h)	1.16±0.17	3.65±1.21	4.87±0.58†	7.06±1.22

*$p<0.01$ between each group.
†$p<0.05$ between each group.

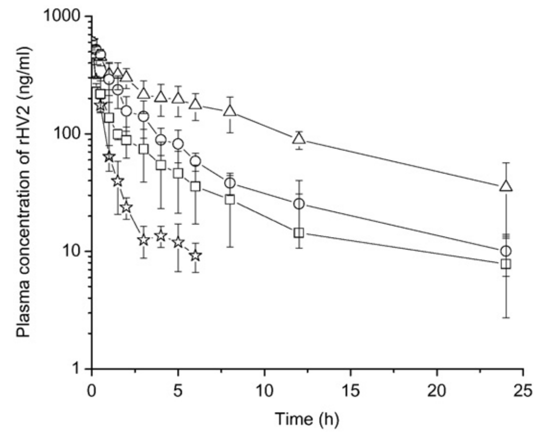

Fig. 5. Plasma rHV2 concentration-time profiles following i.v. injection of rHV2 solution (☆), rHV2-EPC-REVs (□), rHV2-DC-Chol-REVs (○) and rHV2-SA-REVs (Δ). The dose for all the formulations was 0.5 mg/kg. Each value represents mean±S.D. (n=5–6) and the profile is given as a semilogarithmic plot.

3.7. Pharmacodynamic experiments

APTT and TT were used to evaluate the pharmacological efficacy of rHV2 since they represent different mechanisms [29] and reflect different sensitivity. The percentage of changes (vs. normal value) in APTT and TT after i.v. administration of rHV2 formulations at 0.5 mg/kg is shown in Fig. 4.

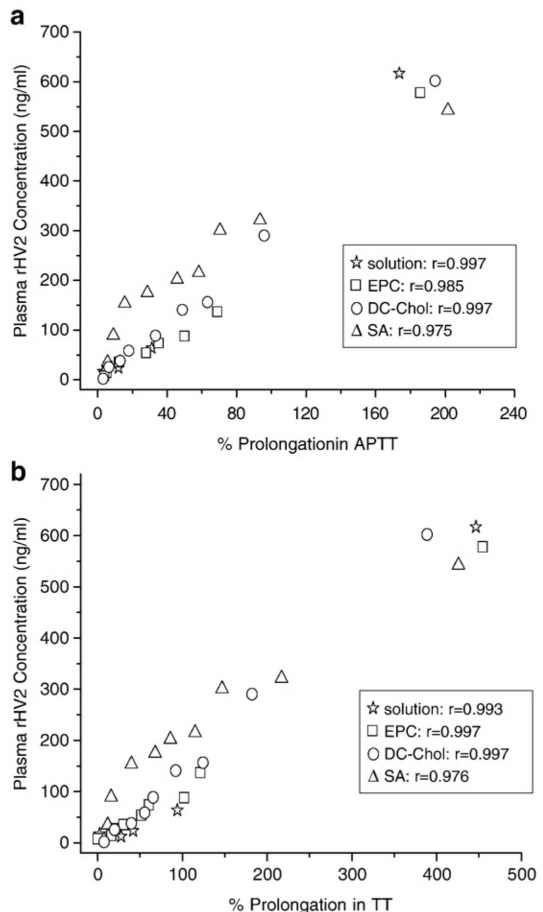

Fig. 6. Relationship between % prolongation in APTT (a), TT (b) and plasma rHV2 levels following i.v. injection of rHV2 solution (☆), rHV2-EPC-REVs (□), rHV2-DC-Chol-REVs (○) and rHV2-SA-REVs (Δ) in five to six rats.

There were no changes of the two parameters for blank liposome groups (data not shown). After i.v. injection of rHV2 solution, the clotting times dropped rapidly due to fast elimination of rHV2. The APTT and TT returned to normal values after 1 h and 2 h, respectively. They were significantly prolonged by all liposomal rHV2 ($p<0.01$), most remarkably for CL groups. The APTT remained above normal value for up to 2 h for rHV2-EPC-REVs, 3 h for rHV2-DC-Chol-REVs and 4 h for rHV2-SA-REVs ($p<0.05$ between EPC and CL groups). The TT prolongation was more obvious in liposome groups and the values finally returned back to normal levels after 4 h and 6 h for EPC and DC-Chol group, respectively. The most prominent prolongation of TT was observed in SA group, in which the value fell back to normal level after 7 h. These results are clearly illustrated in the enlarged part on the top right corner. All these data demonstrate that the rHV2 loaded cationic liposomes are beneficial to improving the pharmacological availability of rHV2.

3.8. Pharmacokinetic experiments

A well-established method, chromogenic thrombin substrate assay [34,38,41] was used to determine plasma concentration of active rHV2.

After i.v. administration of rHV2 formulations (0.5 mg/kg), the C_0 values of 550–600 ng/ml were obtained for all groups (Table 2). The plasma rHV2 level decreased distinctly in solution group, less than 100 ng/ml after 1 h and finally fell below detection limit after 3 h (Fig. 5), with a terminal half-life of 0.71±0.19 h. When an identical dose of rHV2 formulated in liposomes was administered, the plasma rHV2 concentration was maintained above the detection limit for a longer time, in which CL led to extraordinarily higher plasma level of rHV2 than neutral lipids ($p<0.05$). The most elevated plasma rHV2 level was found in SA group and maintained for up to 12 h. The terminal half-life of rHV2 in liposomal groups increased to 2.11±0.19 h for EPC group, 3.52±0.48 h for DC-Chol group and 6.47±1.32 h for SA group.

Therefore, it is concluded that cationic vehicles in this study hold significant advantage in favorable pharmacokinetic behavior of rHV2, in comparison with neutral lipids and solution groups. SA group seems to be the most favorable formulation. The prolonged terminal half-life of rHV2 observed in cationic liposome groups is consistent with previous reports for both protein [16] and DNA drugs [42]. As for zwitterionic group, the pharmacodynamic and pharmacokinetic results of rHV2 were not as satisfactory as expected, which may be caused by low incorporation.

3.9. Preliminary toxicity

During pharmacodynamic and pharmacokinetic studies, all rHV2 formulations were well tolerated in animals tested. No obvious functional changes were observed. No signs of serious toxicity were noted and all the animals survived. It is speculated that the acceptable safety may owe to the low toxicity of rHV2 and the biodegradable properties of the cationic lipids used. Although extensive studies are still needed, the lack of toxicity of DC-Chol was confirmed by Leaf Huang et al. [43,44]. In those studies, the administration of DC-Chol was not related with immunopathology [43] or organ damage [44] after repeated intravenous or arterial injection to pigs and rabbits. Interestingly, DC-Chol also showed anti-inflammation activity, regardless of the administration routes [45,46].

3.10. Relationship between pharmacokinetic and pharmacodynamic results

Correlation between the percentage prolongation of pharmacodynamic parameters and plasma rHV2 levels is shown in Fig. 6. Both APTT and TT prolongation were in good linear relationship with plasma rHV2 levels in four formulations at the doses of 0.5 mg/kg. The satisfactory correlation between APTT prolongation and plasma rHV2 level observed in this study is in good agreement with the result obtained from i.v. injection of rHV2 in dogs [47]. However, the linear correlation between TT prolongation and plasma rHV2 level is inconsistent with published report [47], which is probably caused by the differences in hirudin variant, doses received, plasma assay for rHV2 and/or animal tested.

The rHV2 has a narrow therapeutic window [29], so bleeding events as well as ineffective anticoagulation may occur after stable clotting times are achieved. Therefore, a rapid and easy method is necessary to predict the plasma level of rHV2 in order to avoid overdosing and inadequate anticoagulation. The linear relationship of APTT or TT changes with plasma rHV2 level means that the pharmacokinetic properties of rHV2 formulations can be simply monitored by routine clotting times. Our studies indicate that both pharmacodynamic parameters are reliable in monitoring the anticoagulant effect of free and liposomal rHV2 and predicting plasma level of rHV2 from 10 ng/ml to 700 ng/ml.

4. Conclusion

In summary, cationic lipids, SA and DC-Chol, were demonstrated to greatly increase the EE of rHV2 in SUVs and REVs. The rHV2 loaded cationic liposomes became less positive and relatively stable in serum in comparison with blank cationic liposomes, probably due to electrostatic neutralization induced by negative charge of rHV2. The cationic rHV2-REVs showed sustained-release of rHV2 in the presence of plasma, significantly improved the pharmacological efficacy and enhanced plasma level of rHV2 after i.v. injection in rats compared with neutral lipid group. Both clotting times correlated well with plasma rHV2 concentration. No serious adverse events were noted and physical state of rats was satisfactory for all groups, suggesting the safety of the biodegradable cationic lipids. Electrostatic interaction between negative charge of rHV2 and cationic liposomes was also approved and it might affect all the properties of rHV2 loaded cationic liposomes, including particle size, zeta potential, EE, stability in serum, in vitro release, pharmacodynamic and pharmacokinetic behavior. The present results suggest that cationic liposomes may be a promising sustained-release formulation of rHV2 for i.v. administration and this study would be helpful for controlled delivery of hydrophilic proteins with low pI.

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