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ORIGINAL ARTICLE

Preparation of pegylated lumbrokinase and an evaluation of its thrombolytic activity both *in vitro* and *in vivo*

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KEY WORDS

Lumbrokinase;
Methoxy polyethylene glycol succinimidyl carbonate;
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Abstract Lumbrokinase (LK) is a group of serine proteases with strong fibrinolytic and thrombolytic activities. In clinical practice, LK can only be administered orally because of its antigenicity, immunogenicity and potential to produce anaphylactic reactions after injection. However, many useful drugs such as interferon, insulin, erythropoietin and interleukin have been modified with polyethylene glycol (PEG) to prepare injectable formulations. In this study, LK was modified with methoxy PEG succinimidyl carbonate (mPEG-SC) with molecular weights of 5000, 10,000 and 20,000 and the pegylated products were isolated and purified using the Akta protein purification system. The extent of pegylation was determined by HPLC. Fibrinolytic activities of pegylated and unmodified LK were measured both *in vitro* against urokinase on fibrin plates and *in vivo* using a mouse carageenan black tail model. Optimal pegylation was obtained using mPEG-SC_{5000} in a buffer pH 8.0 with a reaction time of 5 h, reaction temperature of 0 °C and LK:mPEG-SC molar ratio of 1:25. The results show that mPEG modified LK has strong fibrinolytic and thrombolytic activities both *in vitro* and *in vivo*. It is suggested that the pegylated LK is a promising injectable thrombolytic agent for the treatment of thrombotic diseases in clinical practice.

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1. Introduction

Thrombotic diseases are potentially fatal conditions of multiple origins. Various thrombolytic enzymes, such as streptokinase and urokinase, have been used in the treatment of thrombotic diseases along with recombinant tissue plasminogen activators. Lumbrokinase (LK), a group of serine proteases with strong fibrinolytic activity[1,2], can dissolve fibrin directly or activate profibrinolysin to dissolve it indirectly. LK has stimulated considerable interest because of its stability, low cost and facility for oral administration[3–8]. However, thrombotic disease is usually an acute illness for which oral administration is unsuitable because of issues with bioavailability and slow rate of onset[9]. Therefore, an injectable formulation of LK is desirable but brings with it the potential for antigenicity, immunogenicity and anaphylactic reactions. One way to reduce these problems is to introduce structural modifications to LK which do not compromise its bioactivity and resistance to degradation.

Pegylation is a well-developed process that can be applied to therapeutic proteins to enhance their pharmacological activity, increase their half-life, improve their safety and tolerability, reduce their immunogenicity, antigenicity and toxicity and ultimately lead to increased patient compliance and quality of life[10]. Methoxy PEGs (mPEGs) are amphiphilic polymers made up of repeating ethylene oxide subunits with a methoxy group at one end and a terminal hydroxyl group that can be chemically activated at the other[11]. They are available in a variety of configurations and molecular weights and can be linear or branched[10,12]. Succinimidyl carbonate derivatives of mPEGs (mPEG-SCs) were recently introduced for modification and cross-linking of proteins and preparation of high molecular weight polymers.

In this study, the pegylation of LK using different reaction conditions and mPEG-SCs of different molecular weight was investigated and the fibrinolytic and thrombolytic activities of the pegylated LKs were determined both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Crude LK was purchased from Shanghai Guoyuan Bio-tech Co., Ltd. (Shanghai, China). mPEG-SCs with molecular weights of 5000, 10,000 and 20,000 (mPEG-SC_{5000}, mPEG-$SC_{10,000}$ and mPEG-$SC_{20,000}$, respectively) were purchased from Jenkem Technology (Beijing, China). An SDS-PAGE Test Kit was obtained from Beijing Cellchip Biotechnology Co., Ltd. and SDS-PAGE protein standards were purchased from HOUBIO Tech. Co., Ltd. (Hong Kong). Other standard proteins were purchased from the National Institute for Food and Drug Control. All other reagents and chemicals were of analytical grade and used as received.

2.2. Isolation and purification of LK

Crude LK powder was dissolved in 100 mM sodium phosphate at a concentration of 10 mg/mL. Size exclusion chromatography was then performed using the Akta Purify 10 system (GE Company) with a Superdex 75 (HiLoad Superdex 75, 16/60) column and a mobile phase of 50 mM PBS, 150 mM NaCl (pH 7.2) at a flow rate of 0.8 mL/min. Detection was by UV absorption at 280 nm. Two peaks (A and B) were observed in the size exclusion chromatogram (Fig. 1a) of which one (peak B) possessed strong fibrinolytic activity (see Section 2.5). Subsequently the fraction responsible for peak B was subjected to cation exchange chromatography using the same system but equipped with a Hi Trap DEAE FF column (5 mL). Binding was performed with 50 mM PBS (pH 7.2) after which elution involved a linear gradient over 60 min with 50 mM PBS, 1 M NaCl (pH 7.2) at a flow rate of 1 mL/min. Detection was again by UV absorption at 280 nm[13–17]. The cation exchange chromatogram (Fig. 1b) also showed two peaks (C and D) of which one (peak D) showed strong fibrinolytic activity. Molecular weights of the components

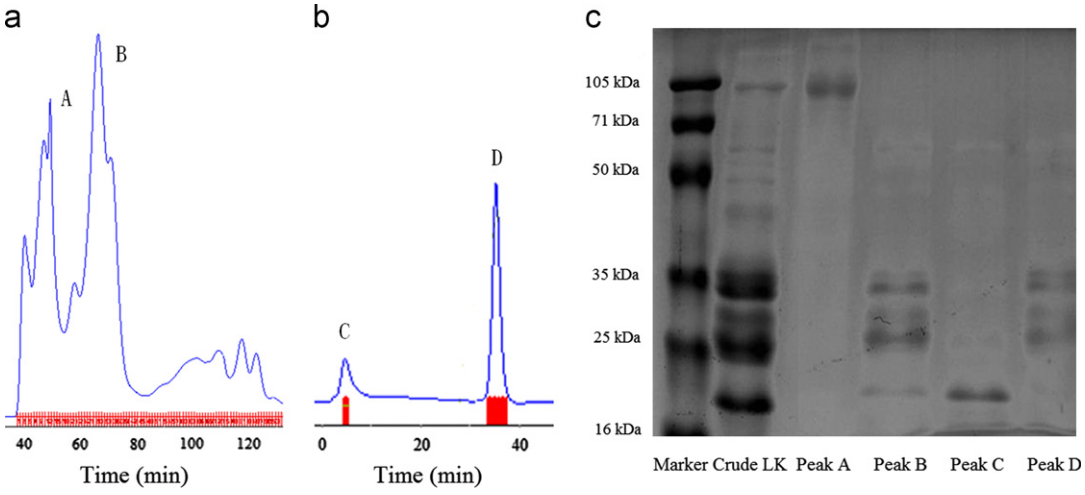


Figure 1 Isolation and purification of crude LK. (a) Size exclusion chromatogram of crude LK. Peak A is some high-molecular weight polymer; peak B has strong fibrinolytic activity. (b) Cation exchange chromatogram of peak B. Peak C is some low-molecular polymer and protective additive; peak D is purified LK. (c) SDS-PAGE (10%, *w*/*v*) of the crude LK and compounds responsible for the four peaks in the process of purification with Akta purify 10. Each band in the SDS-PAGE gels corresponds to each fraction.

from the four peaks were measured by SDS-PAGE using 10% polyacrylamide gradient gel with standard proteins as markers (Fig. 1c). Finally, the crude and purified LK were examined by HPLC (Fig. 2) using the following conditions: Column G-450 (250 mm × 4.6 mm, 5 μm, Agilent); mobile phase 130 mM NaCl, 20 mM PBS (pH 5.0); flow rate 1 mL/min; detection wavelength 280 nm; injection volume 20 μL.

2.3. *Optimization of the pegylation reaction*

2.3.1. *Basic procedure*

The reaction takes place between the primary amino group of LK and the terminal carboxylic group of mPEG-SC in a buffer solution (Scheme 1). Purified LK (3 mg) was mixed with mPEG-SC in a molar ratio of 1:25 in 6 mL 50 mM PBS (pH 7.0) at 4 °C. After 3 h, the reaction was stopped by adding 11 mg/mL glycine[10,18]. All products were subjected to ultrafiltration through a membrane of MW 30,000[19] after which solutions were lyophilized for 48 h to obtain modified LKs. Starting materials and pegylated products were examined by HPLC to calculate the extent of pegylation according to the following equation[19]:

$$\text{Extent of pegylation (\%)} = (1 - \text{residual peak area of LK after modification} / \text{peak area of LK before modification}) \times 100\% \quad (1)$$

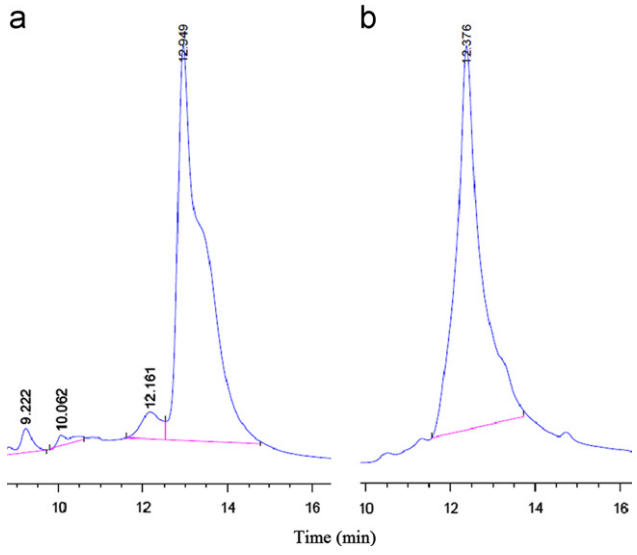


Figure 2 HPLC chromatograms of (a) crude LK and (b) purified LK on a G-450 column (Agilent) using a mobile phase of 130 mM NaCl, 20 mM PBS (pH 5.0) at a flow rate is 1 mL/min and detection at 280 nm. The main peak due to LK is at 12.37 min; the peaks of impurities in (a) elute at 9.22, 10.06 and 12.16 min, respectively.

2.3.2. *Effect of buffer pH*

In this study, 3 mg of purified LK was mixed with mPEG-SC_{5000}, mPEG-$SC_{10,000}$ or mPEG-$SC_{20,000}$ at a molar ratio of 1:5 in 6 mL 50 mM PBS with pH 6.0, 7.0, 8.0 or 9.0 at 4 °C[20–22]. The reaction was stopped after 3 h and products isolated as described above.

2.3.3. *Effect of reaction time*

The reaction was carried out as described in Section 2.3.2 with buffer pH of 8.0. The reaction was stopped after 1, 2, 3, 4, 5 and 6 h and products from 0.5 mL samples were isolated.

2.3.4. *Effect of reaction temperature*

The reaction was as described in Section 2.3.2 with buffer pH of 8.0 and reaction temperatures of either 0, 4 or 25 °C and products isolated.

2.3.5. *Effect of molar ratio*

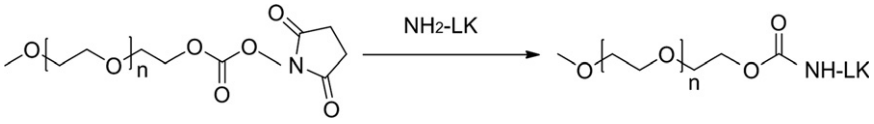
The reaction was as described in Section 2.3.2 with buffer pH 8.0 and the LK:mPEG-SC molar ratio either 1:3, 1:4, 1:5, 1:10, 1:15, 1:20, 1:25 or 1:30 and products isolated.

2.4. *Fibrinolytic activity of LK-mPEG-SC in vitro*

In vitro fibrinolytic activity was measured using the method of Asturp and Mullertz[23] and urokinase as standard. Protein concentration of each enzyme solution was determined by the method of Lowry with BSA as standard. The fibrin agarose plate contained 120 mg agarose, 40 mg bovine plasma fibrinogen and 7 mg thrombin. The clot was allowed to form at room temperature for 1 h after which 10 μL of each sample was carefully transferred onto a plate and incubated for 18 h at 37 °C[24,25]. The diameter of the lytic circle divided by the protein concentration (X) was used to calculate fibrinolytic activity (Y) using the equation $Y = 12{,}737 \ln X + 8614.5$, $R = 0.997$ based on the urokinase standard.

2.5. *Fibrinolytic activity of LK-mPEG-SC5000 in vivo*

Solutions of purified LK and LK-mPEG-SC_{5000} made under optimal conditions were prepared at a concentration of 5 mg/mL. A total of 18 male mice (weight 20 ± 5 g) were randomly assigned to three groups ($n = 6$) to receive intraperitoneal (i.p.) injections of saline (control) or 25 mg/kg purified LK or 25 mg/kg LK-mPEG-SC_{5000} 24 h and 1 h before and 24 h and 48 h after an i.p. injection of carrageenan (20 mg/kg)[26,27]. Carrageenan causes the tail ends of mice to become black due to its thrombolytic activity. At 72 h after the carageenan injection, the length of the black part of the tails of each mouse (the thrombus length) was measured and the average taken as the measure of the thrombotic effect. The *in vivo* study was approved by the Animal Care Ethics Committee of Institute of Materia Medica.



Scheme 1 Pegylated modification of LK by mPEG-SC.

3. Results and discussion

3.1. Isolation and purification of LK

Fig. 1a shows the two peaks in the size exclusion chromatogram of crude LK. Fig. 1b shows the two peaks in the cation exchange chromatogram of peak B. The compound responsible for peak D showed strong fibrinolytic activity and was isolated as purified LK.

Crude LK and the components responsible for peaks A, B, C and D in the purification process were examined by SDS-PAGE. As shown in Fig. 1c, crude LK migrated as several bands, the four main ones having molecular weights of approximately 105, 35, 27 and 20 kDa. Size exclusion chromatography showed peak A to be a single protein with a high molecular weight of about 105 kDa whereas peak B showed three bands with molecular weights of 35, 27 and 20 kDa. As the effective molecular weight of crude LK was given as 25–45 kDa, the results of SDS-PAGE are consistent with the fibrinolytic test, peak A being some polymerized derivative. SDS-PAGE of peaks C and D in the cation exchange chromatogram of peak B revealed peak C migrated as one band with a molecular weight of about 20 kDa while peak D migrated as two bands with molecular weights of about 27 and 35 kDa. Peak C with little or no fibrinolytic activity is probably a proteinaceous preservative agent whereas peak D with strong fibrinolytic activity and a molecular weight consistent with that of pure LK was regarded as the purified sample.

As regards the HPLC study, crude LK gave a single large peak at 12.95 min accompanied by several impurity peaks with retention times of 9.22, 10.06 and 12.16 min (Fig. 2a). Purified LK, on the other hand, showed the same large peak (this time at 12.38 min) with no impurity peaks witnessing to the efficacy of the purification process.

3.2. Optimal pegylation conditions

Purified LK, mPEG-$SC_{20,000}$ and the pegylated product were examined by HPLC. As shown in Fig. 3, the peak of purified LK is again at 12.38 min (Fig. 3a) whereas the peak of mPEG-$SC_{20,000}$ elutes at 13.22 min (Fig. 3b). Both these peaks are present in the HPLC chromatograph of the pegylated product (Fig. 3c) along with two new peaks at 9.62 and 10.34 min presumably resulting from a compound arising from a 1:1 reaction and one from more extensive pegylation[28].

The effect of pH on the pegylation reaction (Fig. 4a) shows that higher pH increases the extent of reaction but decreases protein stability. Pegylation occurred most rapidly at pH 8.0 with LK-mPEG-SC_{5000}, LK-mPEG-$SC_{10,000}$ and LK-mPEG-$SC_{20,000}$ producing 51.35%, 42.04% and 30.22% reaction respectively. Stability of LK was not compromised below pH 8.0 and was used in all subsequent reactions.

The effect of reaction time on pegylation is shown in Fig. 4b. Over the first 3 h, the extent of reaction was relatively constant but increased to some extent in the period 3–5 h. After 5 h, the extent of reaction decreased possibly due to protein instability. On this basis, 5 h was considered to be the optimum duration of reaction.

As regards temperature, Fig. 4c shows that pegylation occurred to the greatest extent at 0 °C and to a lesser extent at the higher temperatures. Thus 0 °C was chosen as the optimum reaction temperature.

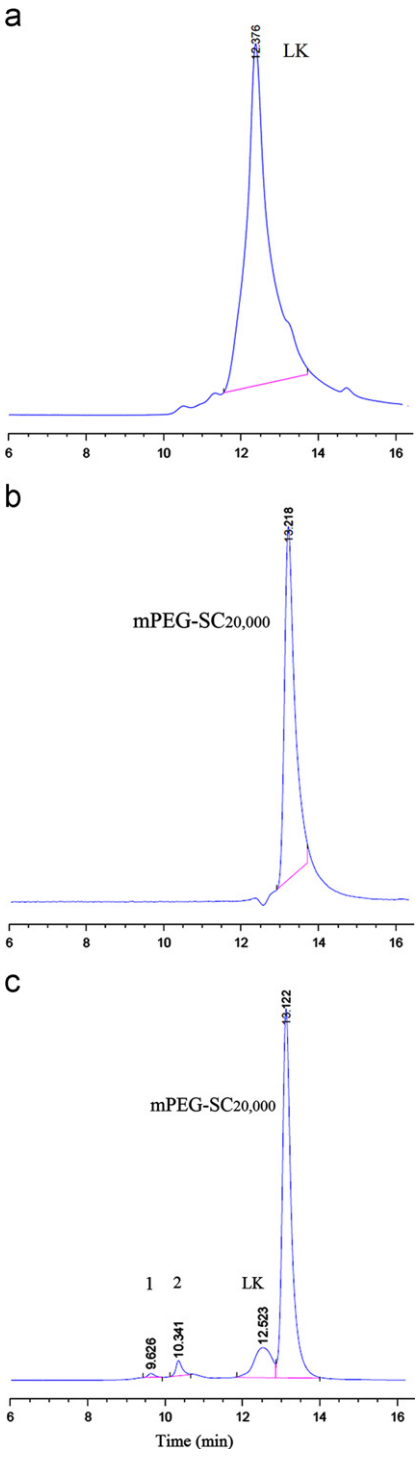


Figure 3 HPLC chromatograms of (a) purified LK, (b) mPEG-$SC_{20,000}$ and (c) the pegylated product using the same conditions as in the Fig. 2 caption. The peak due to LK is at 12.38 min, the peak due to mPEG-$SC_{20,000}$ is at 13.22 min and the pegylated product has new peaks at 9.62 min and 10.34 min.

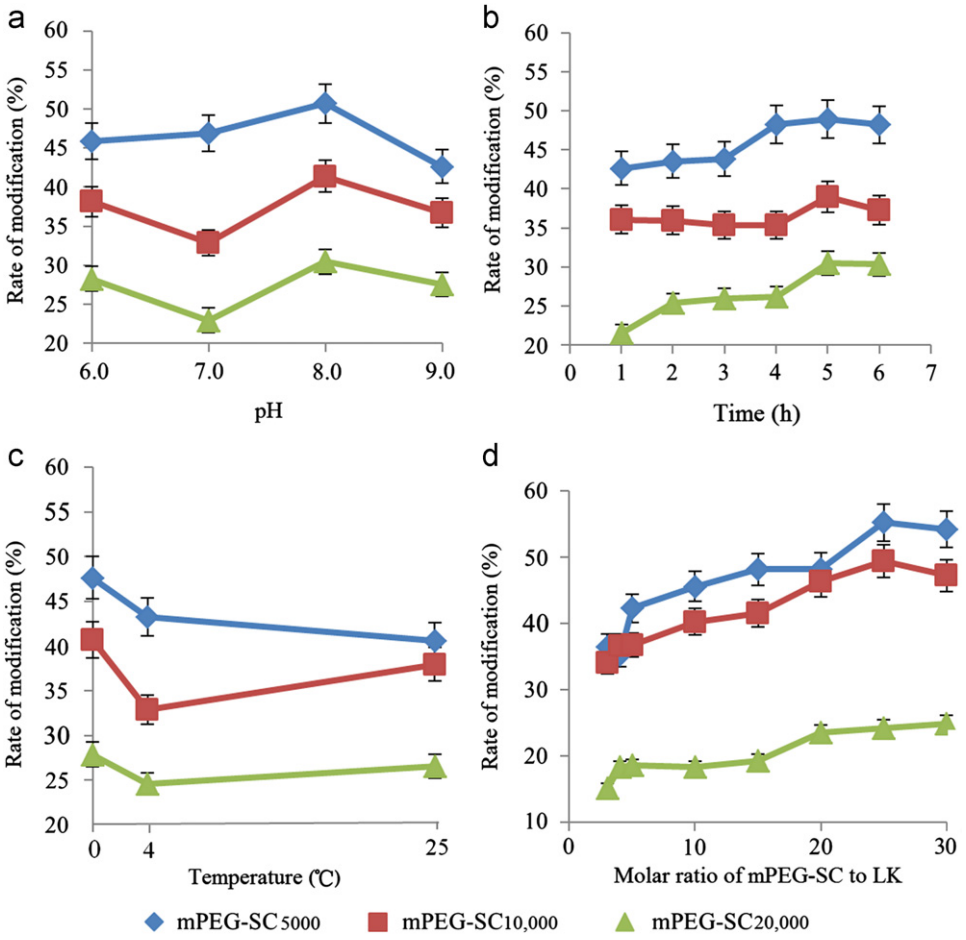


Figure 4 Effect of buffer pH, reaction time, reaction temperature and molar ratio (mPEG-SC to LK) on extent of pegylation. Purified LK was reacted separately with mPEG-SC_{5000}, mPEG-$SC_{10,000}$ or mPEG-$SC_{20,000}$ at (a) different pHs (6.0–9.0), (b) different reaction times (1–6 h), (c) different reaction temperatures (0, 4 and 25 °C) and (d) different molar ratios of LC:mPEG-SC (1:3 to 1:30). The extent of reaction (%) was determined by HPLC.

Considering the molar ratio of the reactants, the extent of pegylation increased with increasing LK:mPEG-SC ratio reaching a maximum at a value of 1:25 and declining at a ratio of 1:30. This indicates mPEG-SC actually impedes the reaction when present at very high concentrations. Based on this result, a molar ratio of 1:25 was chosen as the optimum reaction molar ratio.

3.3. *Fibrinolytic activity of LK-mPEG-SC in vitro*

The lytic circles resulting from the fibrinolytic activities of crude LK, purified LK, LK-mPEG-SC_{5000}, LK-mPEG-$SC_{10,000}$ and LK-mPEG-$SC_{20,000}$ are shown in Fig. 5 and correspond to values (in U/mg protein) of 16,000, 19,995, 12,426, 11,114 and 9869, respectively. The increase in fibrinolytic activity from crude to purified LK shows purification achieves the desired result. However, comparison of the fibrinolytic activity of the pegylated products shows that the activities of all products are lower than that of purified LK although the values are not significantly different. Despite the trend for pegylation to decrease *in vitro* fibrinolytic activity, this may not appertain *in vivo* because of the longer circulation half life of PEG modified LK. Interestingly, since LK-mPEG-SC_{5000} has the highest fibrinolytic activity it appears the higher molecular weight mPEG-SCs hinder the fibrinolytic activity of LK.

3.4. *Fibrinolytic activity of LK-mPEG-SC_{5000} in vivo*

Carrageenans are a family of linear sulfated polysaccharides extracted from red seaweeds. Hu et al.[29] found that carrageenan can be used to establish a mouse thrombolytic model involving blackening of the tail due to local inflammation and endothelial cell damage. As the optimal condition of pegylation was proved to be as follows: pH 8.0, reaction time of 5 h, reaction temperature of 0 °C, molar ratio of mPEG-SC to LK of 1:25, PEG MW of 5000, we selected the optimal condition to develop pegylated LK and evaluated the fibrinolytic activity of LK-mPEG-SC_{5000} *in vivo*. As shown in Fig. 6 and Table 1, the incidence of thrombosis and thrombus length in the groups receiving purified LK and LK-mPEG-SC_{5000} was significantly less than in the control group ($P<0.01$). In addition, the incidence of thrombosis and thrombus length in the LK-mPEG-SC_{5000} group was significantly less than in the LK group ($P<0.05$) indicating that the pegylated product is more

thrombolytic due to its longer half life. As another indicator of efficacy, the tails of mice in the LK-mPEG-SC_{5000} group were lighter in color than in both the purified LK and control groups which were dark black in color (Fig. 6). The fact that pegylated LK has a higher molecular weight than LK presumably protects it from metabolic clearance and masks its immunogenic sites[10]. Whether pegylated LK is in fact less antigenic, immunogenic and anaphylactic than LK will be the subject of a future study.

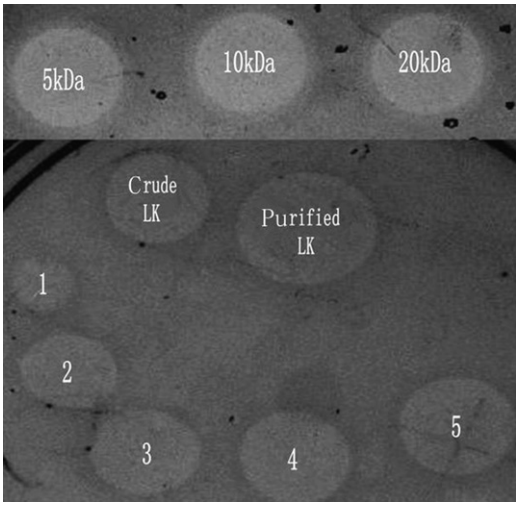


Figure 5 *In vitro* fibrinolytic activity (lytic circles) of crude LK, purified LK, LK-mPEG-SC_{5000} (5 kDa), LK-mPEG-$SC_{10,000}$ (10 kDa) and LK-mPEG-$SC_{20,000}$ (20 kDa) on fibrin plates. All enzymes were dissolved in 50 mM PBS (pH 7.0) at a final concentration of 0.2 mg/mL, applied at a volume of 10 μL and incubated at 37 °C for 18 h. Urokinase (UK) was used as standard at concentrations of (1) 4000 U/mL (2), 8000 U/mL (3), 12,000 U/mL (4), 16,000 U/mL and (5) 20,000 U/mL.

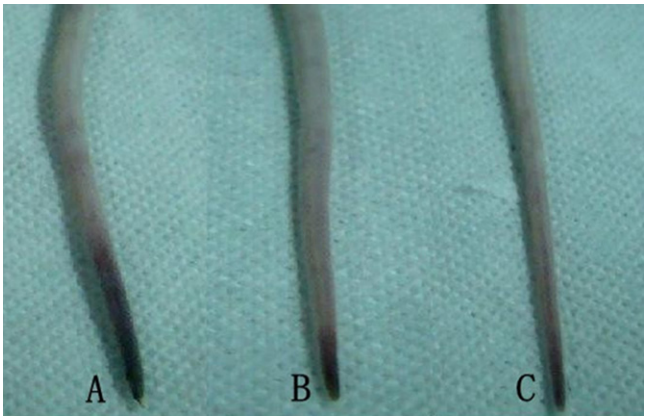


Figure 6 Representative tails from mice 72 h after an intraperitoneal injection of carrageenan (20 mg/kg) and administration of (A) normal saline, (B) purified LK and (C) LK-mPEG-SC_{5000}.

4. Conclusions

The conditions for the successful pegylation of LC by mPEG-SC have been optimized and shown to produce a product with strong fibrinolytic and thrombolytic activities both *in vitro* and *in vivo*. The product produced from mPEG-SC_{5000} is a novel thrombolytic agent that may be clinically useful in the treatment of acute thrombotic disease. The results of this study provide the foundation for further research into the formulation of an injectable LK for human use.

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Table 1 Effect of purified LK and LK-mPEG-SC_{5000} on the incidence and extent of thrombosis in mice ($n=6$).

Group	Dosage (mg/kg)	Incidence of thrombosis (%)	Thrombus length (mm)
Control	–	88	15.8 ± 0.9
Purified LK	25	54[a]	11.8 ± 1.2 [a]
LK-mPEG-SC_{5000}	25	42[a,b]	9.9 ± 1.0 [a,b]

Thrombus length is expressed as mean ± standard deviation.
[a] $P<0.01$ for the purified LK and LK-mPEG-SC_{5000} groups *versus* control.
[b] $P<0.05$ for the LK-mPEG-SC_{5000} group *versus* the purified LK group.

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