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Characterization and Secondary Structure Analysis of Endostatin Covalently Modified by Polyethylene Glycol and Low Molecular Weight Heparin

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Endostatin (ES), as an angiogenesis inhibitor, has been approved by the State Food and Drug Administration (SFDA) in China for the treatment of patients with non-small-cell lung cancer. However, as a protein drug, there are a lot of obstacles on its clinical application, such as need of high dose to maintain its efficacy, expensive and poor stability, etc and limits its clinical use. In order to overcome these shortcomings, we chemically modified ES by polyethylene glycol and low molecular weight heparin (LMWH), respectively. The changes of the secondary structure of the modified products were studied by Fourier transform infrared spectroscopy and Circular dichroism spectra to obtain better ES derivatives. Our study demonstrated that the modified products have a better heat tolerance than ES towards. The result of secondary structure analysis suggests the percentage of β-turn in whole protein is an important factor on the activity and heat stability and ES modified by LMWH can maintain higher activity and its secondary structure.

Key words: chemical modification, endostatin, low molecular weight heparin, polyethylene glycol, stability, structure.

Abbreviations: ATR-FTIR, attenuated total reflectance Fourier transform infrared; ES, endostatin; FTIR, Fourier transform infrared spectroscopy; HUVEC, human umbilical vein endothelial cell; KRS-5, thallium bromoiodide; LMWH, low molecular weight heparin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; PEG, polyethylene glycol; TNBS, 2,4,6-trinitrobenzene sulphonic acid.

Tumour vasculature plays a major role in tumour progression and metastasis. Anti-angiogenesis has merged as an important target in cancer therapy and it has generated a great deal of interest in anti-cancer drug discovery and development. So far, several angiogenesis inhibitors have been approved by Food and Drug Administration (FDA) in the US. Endostatin (ES), a potent endogenous angiogenesis inhibitor, was found in 1997 (*1*). As a new type of potent anti-cancer agent, it provided a novel and efficient way for cancer treatment (*2*, *3*). ES can effectively inhibit angiogenesis, so it can inhibit the growth and metastasis of tumours. ES also decreased drug resistance in long-term and repeated treatment when it used in combination with other chemotherapeutic agents. In September 2005, ES, as an angiogenesis inhibitor, was approved by the State Food and Drug Administration (SFDA) in China for the treatment of patients with non-small-cell lung cancer (*4*). However, there are still lots of obstacles on its clinical application (*5*), such as need of high dose to maintain its efficacy (*6*, *7*), short half-life, poor stability, expensive and some other shortcomings just like other protein drugs.

Recently, chemical modification of protein have been widely studied in order to overcome these obstacles and improve the effectiveness of these drugs, such as prolonging the half-life, lowering the immunogenicity and increasing the stability, etc. Currently, the most commonly used modifier is polyethylene glycol (PEG). In order to assign the activities or endue synergistic effect of bioactive modifiers, we chemically modified ES by PEG (*8*) and low molecular weight heparin (LMWH) (*9*), respectively. In this study, the modification was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), free amino group determination and the test of inhibitory effect on human umbilical vein endothelial cell (HUVEC) proliferation *in vitro*. Heat stability and the secondary structure of ES, PEG–ES and LMWH–ES were also studied.

MATERIALS AND METHODS

Reagents—LMWH (4–5 kD) was purchased from Dongcheng Biochemical Co. Ltd (China). PEG-6000 was purchased from Bio Basic Inc. (USA). Protein molecular weight standard (14.4–94.0 kD) was obtained from Tiangen Biotech Co. Ltd (China). *Pichia* yeast strain containing human ES gene was kindly provided by the Institute of Immunology, Medical School of Shandong University (Jinan, China).

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Preparation of ES—ES was expressed by *Pichia* yeast containing human ES gene. The culture supernatant of *Pichia* yeast was purified by carboxymethylcellulose-II (CM-II, Whatman, USA) ion exchange column chromatography and Sephadex G-50 (Pharmacia, Sweden) column chromatography. The resulting ES exhibited one protein band on SDS–PAGE at the position of 20 kD.

Preparation of PEG–ES Conjugate—PEG-6000 was activated by cyanuric chloride (*10*). The activated PEG-6000 and ES were dissolved in sodium tetraborate buffer (10 mM, pH 9.0) in a molar ratio of 40 : 1. The reaction was carried out at 4°C under slow agitation. During the reaction, aliquots of the solution were pipetted out at 1, 2, 4, 8, 12, 24 and 48 h for SDS–PAGE, free amino group analysis and activity assay, respectively. Then, glycine was added to end the reaction (*11*). The reaction solution was subjected to CM-II ion exchange column and sephadex G-50 column for chromatography purification.

Preparation of LMWH–ES Conjugate—ES was dissolved in 0.3 M sodium carbonate buffer (pH 9.5) and added LMWH activated by periodate oxidation (*12*), then, the solution was agitated slowly in dark at 4°C for 48 h. During the reaction, aliquots of the solution were pipetted out at 1, 2, 4, 8, 12, 24 and 48 h, respectively, for analysis. The reaction was terminated by adding glycine (*13*). The solution was subjected to CM-II ion exchange column and Sephadex G-50 column for chromatography as stated above. The eluent containing LMWH–ES was collected, desalted, concentrated and lyophilized.

SDS–PAGE—Samples taken at 0, 1, 2, 4, 8, 12, 24 and 48 h were analysed by SDS–PAGE at ambient temperature. Electrophoresis was performed according to the method described by Li *et al*. (*14*). In the SDS–PAGE, a discontinuous system was employed, the total monomer concentration of the stacking gel was 3% (w/v) and the separation gel was 15% (w/v). Dimensions of the gel immersed in running buffer (0.1% SDS, 0.05 M Tris/0.384 M glycine buffer, pH 8.3) were 8 cm × 8 cm × 0.1 cm. Samples in loading buffer (1% SDS, 1% mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 0.01 M Tris–HCl, pH 8.0) were incubated at 100°C for 5 min before electrophoresis. Electrophoresis was programmed to a two-step mode with applying constant current of 10 mA in stacking gel and 20 mA in the separation gel. After ~2 h, the gels were stained with coomassie brilliant blue R-250 (Fluka, Germany) overnight and were then destained.

Free Amino Group Determination—The free amino group was determined by 2,4,6-trinitrobenzene sulphonic acid (TNBS) method (*15*). To 1 ml of protein solution (0.6–1 mg/ml) were added 1 ml of 4% $NaHCO_3$, pH8.5 and 1 ml of 0.1% TNBS. The solution was allowed to react at 40°C for 2 h. Then 1 ml 10% SDS was added to solubilize the protein and prevent its precipitation on addition of 0.5 ml 1 mol/l HCl. The absorbance of the solution was read at 335 nm against a blank treated as above but with 1 ml of water instead of the protein solution. Absorbance before (A_0) and absorbance after (A_X) different time reaction were read. $A_X/A_0 \times 100\%$ was the percentage of remaining free amino group and $(1 - A_X/A_0) \times 100\%$ was the degree of free amino group modification.

Activity Assay—The activity of ES and its modified derivatives were assayed by testing the ability in inhibiting HUVEC proliferation *in vitro* using MTT (Sigma, USA) colorimetric assay (*16*, *17*). Log phase endothelial cells were collected and plated in 96-well plate format at 1.0×10^4 cells/well, in a volume of 200 μl. Then cells were incubated at 37°C with humidified 95% air/5% CO_2. After 100 μg/ml ES, PEG–ES and LMWH–ES were added into the wells separately, the cells were incubated for 48 h at 37°C in RPMI 1640 media with 10% calf serum. Then the supernatant was removed and cells were washed with PBS twice. After that, the cells were resuspended in RPMI 1640 media with 10% calf serum and incubated for 4 h at 37°C with 20 μl of 5 mg/ml MTT solution in each well. After the incubation, the supernatant was removed, 150 μl of DMSO was added to each well and the plate was agitated for 10 min. Then the absorbances at 570 nm were read and the inhibition ratios were calculated.

Heat Stability Determination—The unmodified ES, PEG–ES and LMWH–ES conjugates were dissolved in 0.2 M phosphate buffer (pH 7.0) and incubated at 25 or 37°C for 1 h. Samples were taken at 0, 10, 20, 30, 40, 50, 60 min and their activities were determined with the method described above. Their percentages of retained activity were calculated.

Secondary Structure Analysis—The secondary structure of ES, PEG–ES and LMWH–ES were analysed by Fourier transform infrared spectroscopy (FTIR) and Circular dichroism (CD) spectra. For FTIR, solution (of certain concentration) of ES, LMWH–ES and PEG–ES were separately dropped on the centre of thallium bromoiodide (KRS-5) plate, which were then covered by another KRS-5 plate. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were acquired using a Nicolet Nexus 470 FTIR spectrometre equipped with a Smart Endurance diamond ATR accessory (200 scans, 4/cm resolution, the wavenumber range of 4,000–400/cm), at room temperature. The amide I band (1,700–1,600/cm) of ATR-FTIR was transformed to Fourier self-deconvolution spectrum and second derivative spectrum. Spectral manipulations were performed using the spectral analysis software GRAMS/AI (7.02, Galactic Industries Corp., Salem, NH) (*18*).

CD of ES, PEG–ES and LMWH–ES were measured on an Applied Photophysics Chirascan spectropolarimetre using 0.1 mm path length cell at room temperature, with the condition as follow: bandwidth is 1 nm; time-per-point is 1 s. Proteins were dissolved in 50 mM sodium phosphate, pH 7.4 at the final concentration of 0.15–0.2 mg/ml and filtered with 0.45 mm pore size filter unit before use. Protein concentration was measured using Lowry method. The data were treated by CDNN software.

RESULTS

Solubility—The final solution of ES, PEG–ES and LMWH–ES in the soluble protocol did not contain any visible precipitants or fine particles and remained solubilized either at 4°C or after melting from −20°C storage.

SDS–PAGE of PEG–ES and LMWH–ES Conjugates—The molecular weight of ES was characterized by SDS–PAGE, and a single band was shown at the position of 20 kD (Fig. 1A). PEG–ES and LMWH–ES conjugates of different reaction time presented heterogeneity on SDS–PAGE, and protein bands near 38 and 31 kD showed increasing density with prolonging reaction time (Fig. 1C and D). After purification, homogeneous fractions of modified products were obtained separately. Purified PEG–ES and LMWH–ES conjugates presented single band near 38 and 31 kD separately (Fig. 1B).

Modification Effects on the Activity of ES at Different Reaction Time—During modification by PEG, it was found that the activity of ES decreased slightly during 1–4 h and further decreased during 8–24 h and the activity decreased dramatically after 24 h (Fig. 2A). In the modification with LMWH, the activity of ES remained unchanged in the first 1 h reaction. After that, the activity of ES decreased slightly over times and there was a platform period during 12–24 h (Fig. 2B).

Free Amino Group Determination—Free amino group modification increased when the time of modification prolonged and it corresponded to the changes of activity. In the preparation of PEG–ES, the free amino group modification upgraded quickly during 12–24 h. It also increased fast during 24–48 h, but the activity of ES

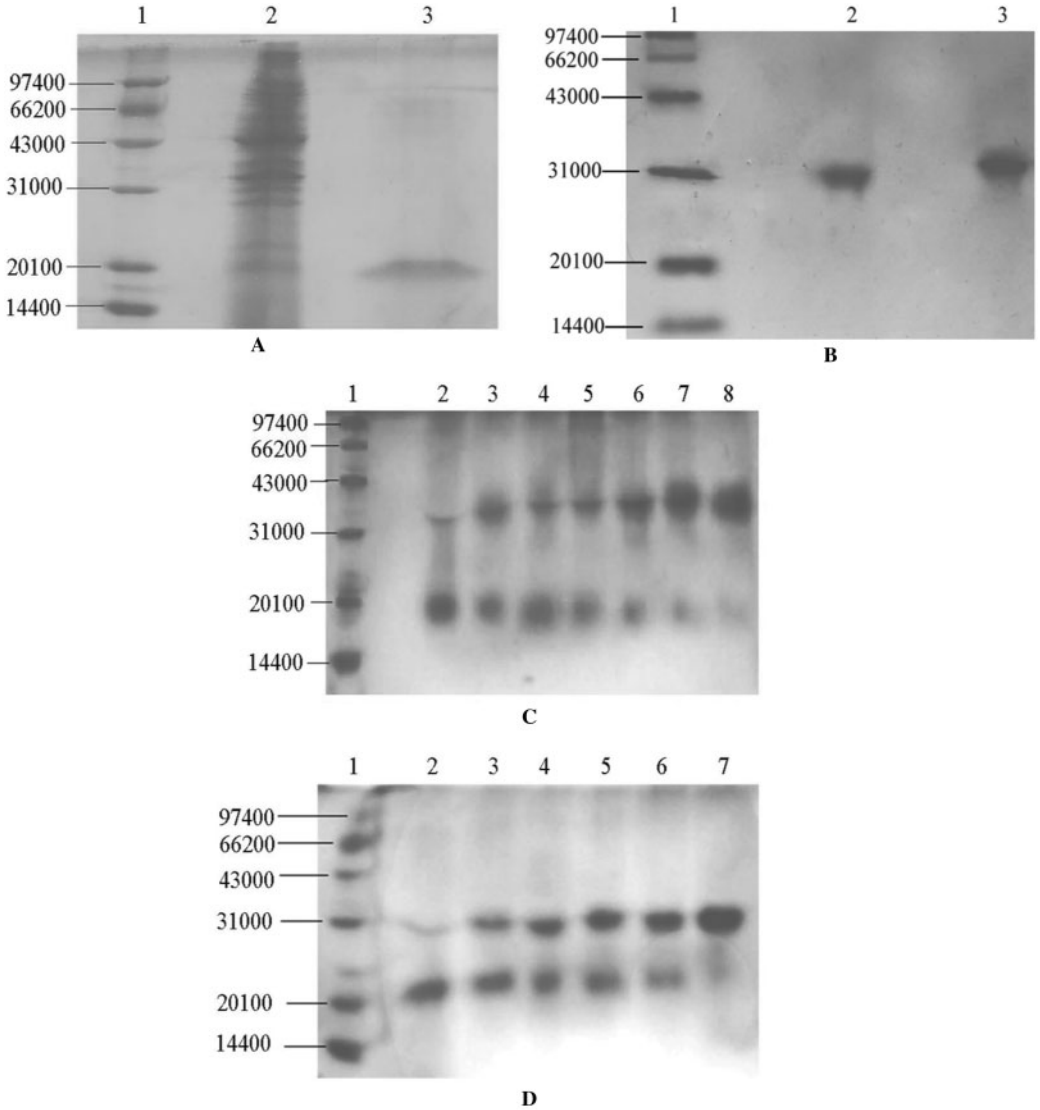

Fig. 1. **SDS–PAGE analysis of unmodified ES, PEG–ES conjugate and LMWH–ES conjugate.** (A) 1, markers; 2, culture supernatant of *Pichia* yeast containing human ES gene; 3, unmodified ES. (B) PEG–ES conjugates with different reaction times: 1, markers; 2–8, samples modified for 1, 2, 4, 8, 12, 24 and 48 h. (C) LMWH–ES conjugates: 1, markers; 2–7, samples incubated for 2, 4, 8, 12, 24, and 48 h. In the SDS–PAGE, a discontinuous system was employed, the total monomer concentration of the stacking gel was 3% (w/v) and the separation gel was 15% (w/v). Dimensions of the gel immersed in running buffer (0.1% SDS, 0.05 M Tris/0.384 M glycine buffer, pH 8.3) were 8 cm × 8 cm × 0.1 cm. Samples in loading buffer (1% SDS, 1% mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 0.01 M Tris–HCl, pH 8.0) were incubated at 100°C for 5 min before electrophoresis. Electrophoresis was programmed to a two-step mode with applying constant current of 10 mA in stacking gel and 20 mA in the separation gel.

decreased significantly (Fig. 2A). In the preparation of LMWH–ES, the free amino group modification increased when the time of reaction prolonged, but the activity decrease was not as dramatic as that of PEG–ES preparation (Fig. 2B).

Heat Stability of PEG–ES and LMWH–ES Conjugates—The effects of heat on the activities of PEG–ES, LMWH–ES conjugates and unmodified ES were evaluated by incubating them at 25 or 37°C. The results are shown in Fig. 3A (25°C) and Fig. 3B (37°C) indicated that the modified ES is more tolerable than native ES and PEG–ES is better than LMWH–ES towards heat treatment.

Secondary Structure Analysis—The amide I band (1,700–1,600/cm) of FTIR was transformed to Fourier self-deconvolution spectrum and second derivative spectrum. The original spectrum, Fourier self-deconvolution spectrum and second derivative spectrum of amide I band (1,700–1,600/cm) of ES, PEG–ES and LMWH–ES were demonstrated (Fig. 4). The spectrum was treated by GRAMS/AI software to get curve fitting spectrum (Fig. 5). Peak position of the amide I band of ES, PEG–ES and LMWH–ES and the relative percentage of conformation was indicated in Table 1 separately. CD spectra of ES, PEG–ES and LMWH–ES were demonstrated (Fig. 6). The data were treated by CDNN software, and the relative percentage of conformation was indicated in Table 2 separately. Both the data of ES by FTIR and CD were similar with the pervious report on recombinant ES (*19*).

The results of relative percentage of conformation demonstrated that the percentage of β-turn of PEG–ES

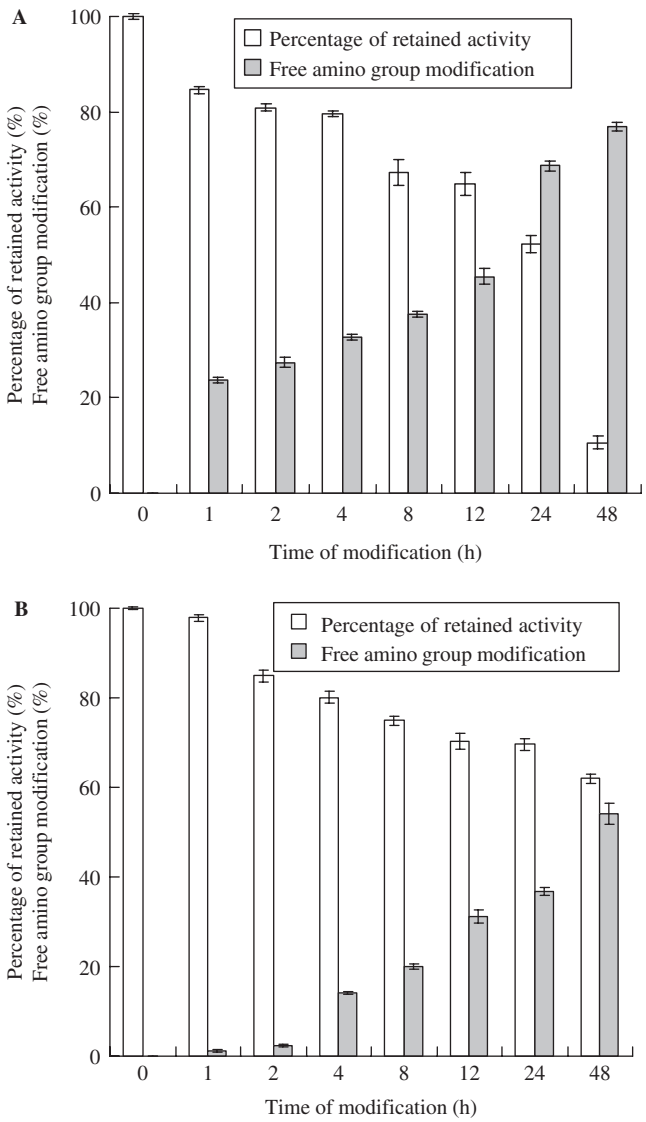

Fig. 2. **Effects of activity of ES with different times of reaction and the degree of free amino group modification of PEG–ES (A) and LMWH–ES (B).** The activity was assayed by testing the ability in inhibiting HUVEC proliferation *in vitro* using MTT colorimetric assay. The free amino group was determined by TNBS method.

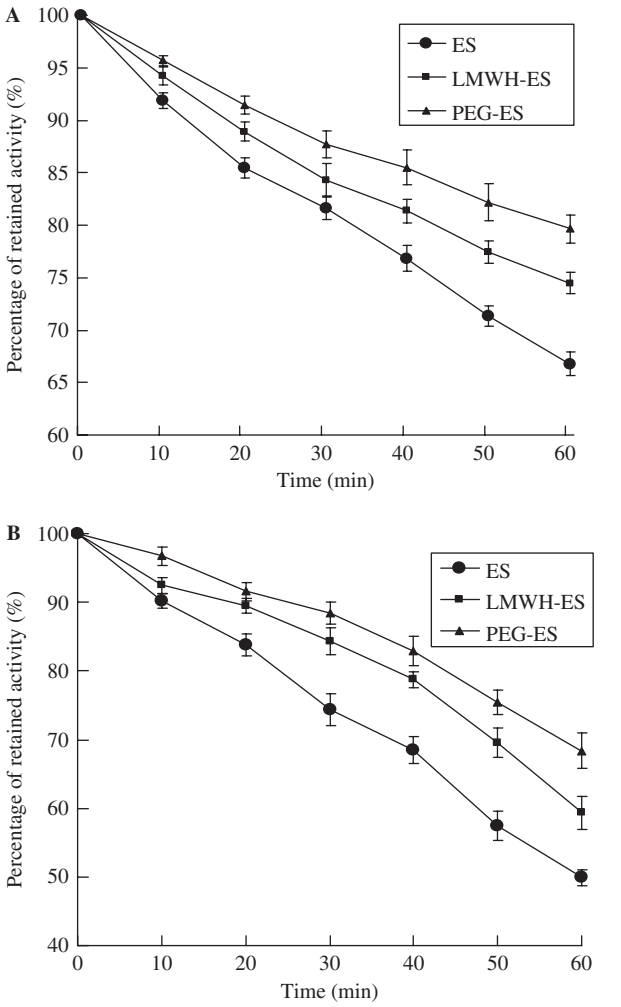

Fig. 3. **Heat stability of ES, PEG–ES conjugates and LMWH–ES conjugates.** (A) Incubation at 25°C; (B) Incubation at 37°C. ES, PEG–ES conjugates and LMWH–ES conjugates were dissolved in 0.2 M phosphate buffer (pH 7.0) and incubated at 25 or 37°C for 1 h. Samples were taken at 0, 10, 20, 30, 40, 50, 60 min and their activities were determined by MTT method. The concentration of ES, PEG–ES and LMWH–ES were all 100 μg/ml.

changed much more obviously than any other secondary structure components. The change of relative percentage of conformation of LMWH–ES was not obvious compared with that of ES.

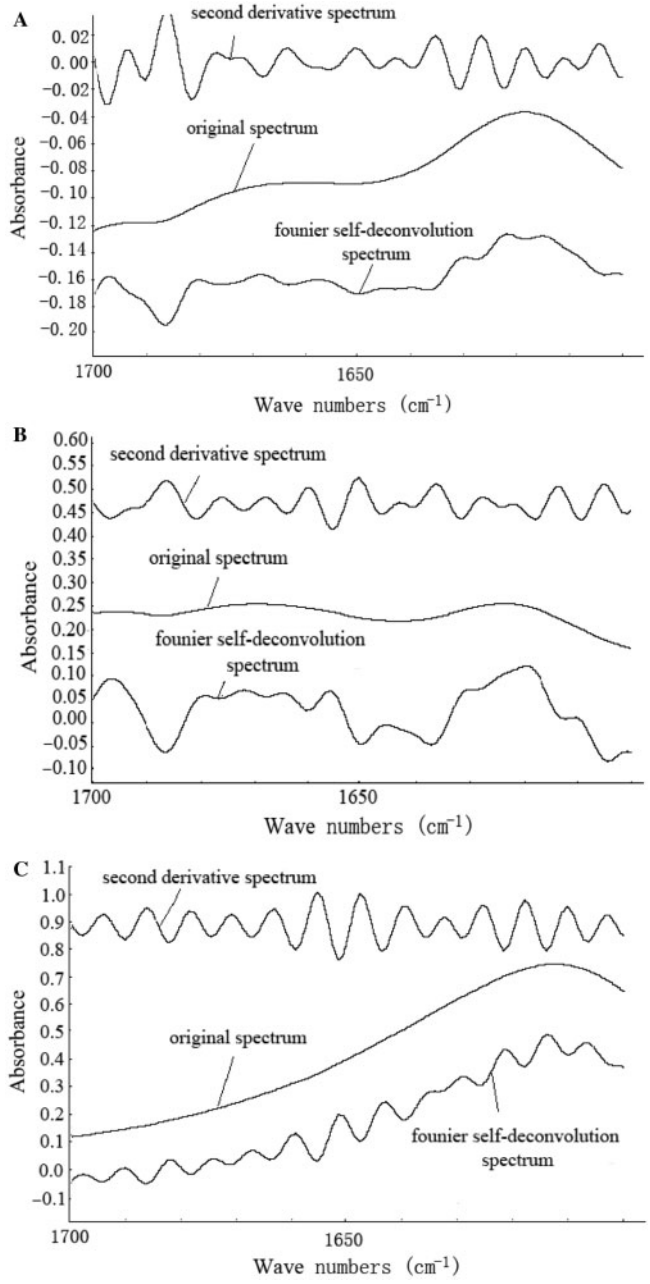

Fig. 4. **Original spectrum, Fourier self-deconvolution spectrum and second derivative spectrum.** (A) ES; (B) PEG–ES (reacted for 24 h); (C) LMWH–ES (reacted for 48 h). ATR-FTIR spectra (original spectra) were obtained by putting sample solutions of certain concentration in a KRS-5 cell and scanning on a Nicolet Nexus 470 FTIR spectrometre equipped with a Smart Endurance diamond ATR accessory (200 scans, 4/cm resolution, wave number range 4,000–400/cm), at room temperature. The amide I band (1,700–1,600/cm) of ATR-FTIR was transformed to Fourier self-deconvolution spectrum and second derivative spectrum.

DISCUSSION

In this study, we modified ES by PEG and LMWH for the first time. PEG has been widely used in modifying proteins and peptides in recent years (*20*, *21*) and its biocompatibility has been approved from the FDA of the US. There are many advantages for PEG modified ES over original ES, such as its lower toxicity, no antigenicity and high solubility, so PEG was chosen to modify ES in this study. LMWH is a kind of polysaccharides with anti-angiogenesis and anti-tumour

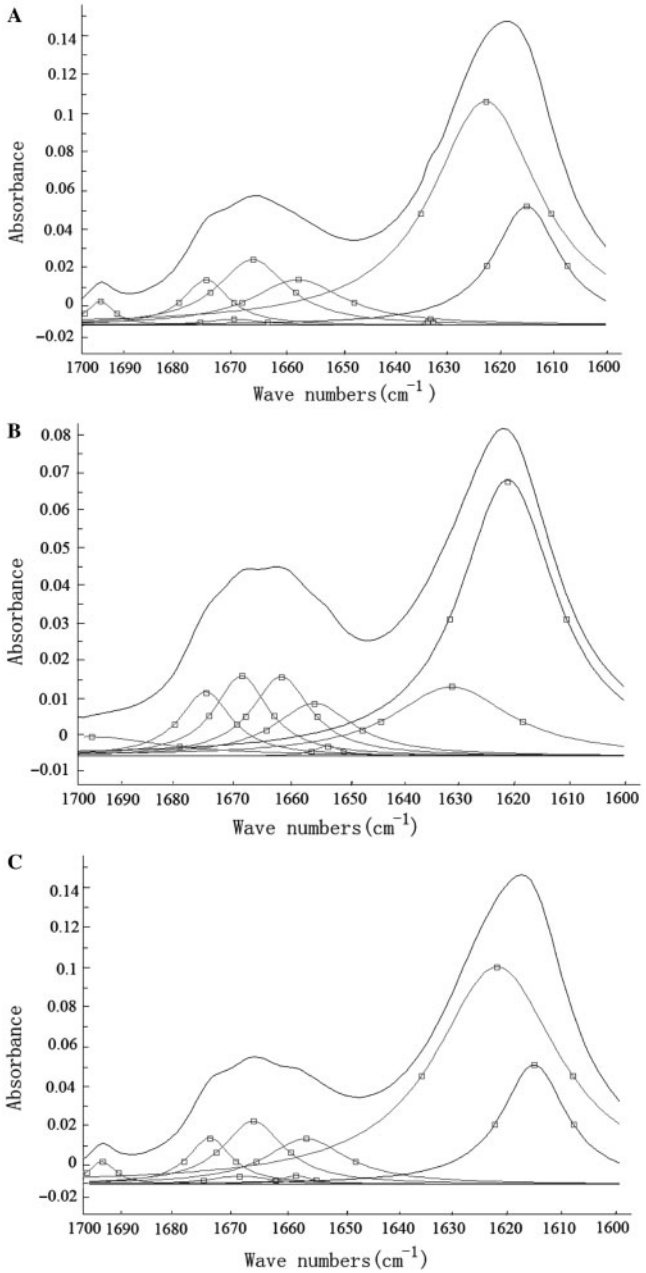

Fig. 5. **Curve fitting spectrum of amide I band (A) ES; (B) PEG–ES; (C) LMWH–ES.** Spectral manipulations were performed using the spectral analysis software GRAMS/AI to get the curve fitting spectra.

Table 1. **Secondary structure analysis of ES, PEG–ES and LMWH–ES of FTIR.**

	ES		PEG–ES		LMWH–ES	
	Peak position (per cm^{-1})	Relative percentage (%)	Peak position (per cm^{-1})	Relative percentage (%)	Peak position (per cm^{-1})	Relative percentage (%)
β-antiparallel	1,696.3[a]	1.32[b]	1,697.3[a]	3.29[b]	1,696.2[a]	1.20[b]
	1,676.3[a]		1,676.6[a]		1,676.4[a]	
β-turn	1,671.0[a]	17.33[b]	1,670.1[a]	26.08[b]	1,670.9[a]	16.19[b]
	1,667.5[a]		1,662.9[a]		1,668.4[a]	
			1,656.9[a]		1,660.6[a]	
α-helix	1,658.9[a]	9.99[b]		9.11[b]	1,658.6[a]	8.85[b]
			1,654.5[a]			
	1,623.4[a]		1,632.0[a]		1,623.3[a]	
β-sheet		71.37[b]		66.58[b]		73.76[b]
	1,615.6[a]		1,621.8[a]		1,616.5[a]	

[a]Signal assignment: 1,615–1,640/cm^{-1}, β-sheet; 1,640–1,650/cm^{-1}, random coil; 1,650–1,660/cm^{-1}, α-helix; 1,660–1,680/cm^{-1}, β-turn; 1,680–1,695/cm^{-1}, β-antiparallel. [b]Relative percentage of conformation calculating:
$\text{Relative percentage}\,(100\%) = \frac{\text{Branch peak area}}{\text{Total peak area of brand I}} \times 100\%$.

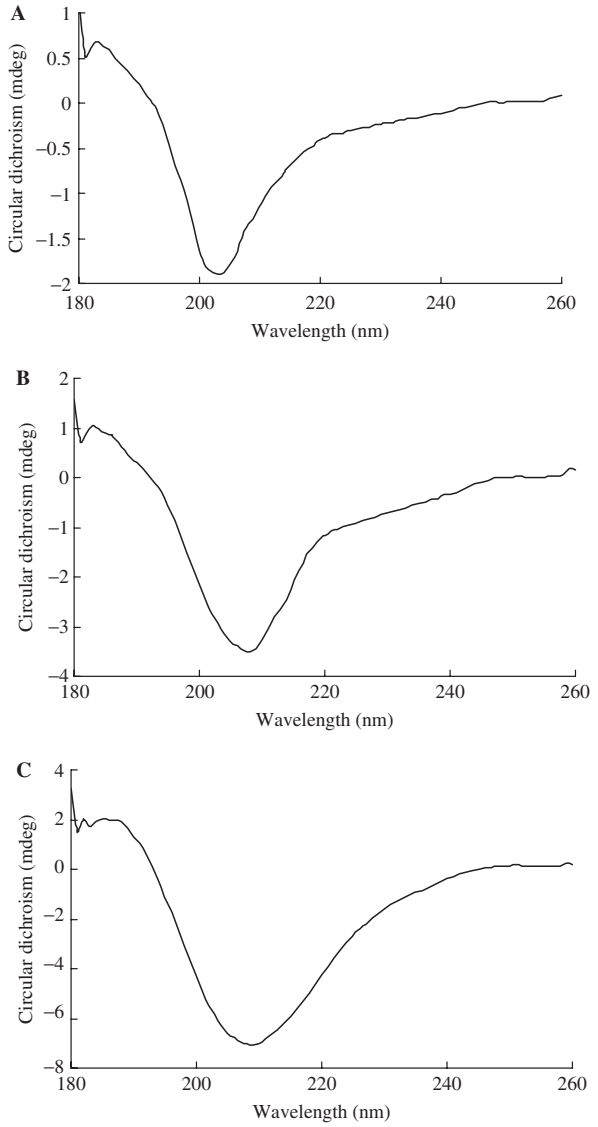

Fig. 6. **CD spectra of ES, PEG–ES and LMWH–ES: CD spectra in 50 mM sodium phosphate, pH 7.4, at room temperature.** (A) ES; (B) PEG–ES; (C) LMWH–ES.

Table 2. **Relative percentage of conformation of ES, PEG–ES and LMWH–ES on CD.**

	Relative percentage (%)		
	ES	PEG–ES	LMWH–ES
β-antiparallel	1.17	1.18	1.45
β-turn	16.2	23.21	16.2
α-helix	11.9	10.24	9.79
β-sheet	70.2	65.37	72.56

activity (*22*), and it also has been used to modify proteins. In our experiment, LMWH was employed to modify ES in order to achieve a synergistic effect and make comparison with the effect of PEG.

SDS–PAGE showed that the molecular weight of PEG–ES was 38 kD. Because the modifier was PEG-6000 and the molecular weight of ES is 20 kD, so we presumed that one molecule ES conjugated with three molecules of PEG-6000 in average. Free amino group modification could achieve up to 68.67% at 24 h of reaction time and its activity could retain >50%. Therefore, we consider 24 h as the ideal reaction time (Fig. 2A). For the similar reason, the ideal reaction time of modification by LMWH was 48 h (Fig. 2B). SDS–PAGE demonstrated that there was a band at 31 kD after modification by LMWH. The average molecular weight of LMWH was 4 kD, the result indicated that one molecule ES conjugated with three molecules of LMWH in average, which coincided with the result of the modification by PEG. The results of activity assay indicated that the activity of ES was affected by prolonging reaction times when it was modified by both PEG and LMWH. These may be two possible reasons, the first one is that the structure may change after modification; and the second one is that the reaction was carried out at 4°C for a certain time and the activity of protein itself may change under this condition.

X-ray crystal structure of ES is already reported (*23*), but because of the huge difficulty to obtain single crystal after combination with a high molecular material such as PEG or LMWH, the X-ray crystal structure of PEG–ES and LMWH–ES have not been obtained, but for the research on the structure after modification,

the secondary structure were analysed by FTIR and CD in this study. The secondary structure analysis by FTIR showed that the secondary structure of PEG–ES was composed of 9.11% α-helix, 3.29% β-antiparallel, 26.08% β-turn and 66.58% β-sheet and that of ES was composed of 9.99% α-helix, 1.32% β-antiparallel, 17.33% β-turn and 71.37% β-sheet. We can see that modification mainly influenced the percentage of β-turn, because it had a large change compared with ES. The secondary structure of LMWH–ES was composed of 8.85% α-helix, 1.20% β-antiparallel, 16.19% β-turn and 73.76% β-sheet. The change on the secondary structure of LMWH–ES was not obvious, and LMWH–ES remained a higher activity than PEG–ES. The analysis of the CD data has a similar result with that of FTIR. In both of these two reactions, the activity decreased with the increase of reaction time (free amino group modification), so β-turn was presumed to play the main role for maintaining ES activity in secondary structure.

In conclusion, we first time carried out successfully covalent modification of ES by PEG, and LMWH. The data reported herein demonstrated that the modified ES conjugates can be monitored by SDS–PAGE, free amino group analysis and the test of inhibitory effect on HUVEC proliferation *in vitro*. Both of these two novel derivatives have better heat stability than ES, but the secondary structure of LMWH–ES has smaller changes compared with that of ES (less change of β-turn) and the changes of activity less than PEG–ES. Therefore, LMWH–ES may be a better derivative of ES and an ideal candidate for preclinical and clinical development. Studies are under way to confirm the anti-tumour efficacy, immunogenicity and toxicity of LMWH–ES and PEG–ES when they were used alone or in combination with chemotherapeutic agents in animal model systems.

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