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Study on Hemostatic Effect of Thrombin Embedded with Different Concentration of Polypeptide RDAD16

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Abstract

Limiting blood loss following trauma is critical for survival and can reduce the risk of death from coagulopathy, infection, and multisystem organ failure. A hemostatic material with biocompatibility and effective hemostatic properties was developed to avoid blood loss. The thrombin spray was prepared by using the polypeptide RDAD16 as a stabilizer. The circular dichroism study showed that the chemical structure of the polypeptide did not change as a stabilizer. The atomic force microscope analysis showed that RDAD16 has formed a fibrous substance in the aqueous solution and could effectively encapsulate the thrombin. The simple coagulation experiment showed that the polypeptide RDAD16 could be the effective stability of the thrombin. The coagulant time of thrombin encapsulated by polypeptide RDAD16 was 14 s after 30 days which showed a significant difference compared with the control sample. Wound closure test in mice showed that thrombin embedded with Polypeptide RDAD16 does not affect wound closure.

Keywords

Thrombin, RDAD16, Coagulation

1. Introduction

Thrombin is a commonly used rapid effective hemostat. Its function is to assist the final step of the physiological coagulation cascade, rapidly change the fibrinogen into a reticular and deposited fibrin, thus it can form a stable blood clot rich in fibrin to achieve rapid hemostasis. As a kind of enzyme reagent, thrombin is deactivated with poor stability, which limits its application. Some studies showed that the aqueous solution of thrombin was completely inactivated after

24 h at normal temperature. There are four main methods to immobilize the enzyme. The carrier binding method, according to the interaction of the enzyme and carrier, can be divided into physical adsorption, ion binding and covalent binding. The combined forces are particle adsorption, charge, covalent bond, etc. Among them, the covalent combination method is the most used. It is an effective method to improve the stability of enzymes by immobilization of enzymes through covalent bonds on substrates. Some studies on the immobilization of thrombin have been carried out [1] [2] [3] [4] to make the retention time of enzyme activity. Crosslinking method: this method is covalent immobilized with a covalent binding method. The difference is that it does not use the carrier. The amino acid residues of the enzyme proteins involved in cross-linking reaction includes amino group, phenol group and imidazole group. The main crosslinking agents are glutaraldehyde, isocyanic acid and double azidine. The reaction conditions of crosslinking reaction are relatively intense. Many groups of enzyme molecules are cross-linked, so the recovery of activity of the immobilized enzymes is generally low. Embedding method: embedding method can be divided into gel embedding method and microcapsule embedding method. It is generally a physical method and does not need to react with amino acid residues of enzyme proteins, so it is widely used. The advantage of immobilized enzymes in physical methods is that enzymes do not participate in chemical reactions. The overall structure remains unchanged, and the catalytic activity of the enzyme is well preserved. However, because the entrapped or semi-permeable membrane has some spatial or three-dimensional hindrance, it is not applicable to some reactions. Immobilization of thrombin on nanoparticles can be seen as a further development of microencapsulation. The main factors affecting the kinetic characteristics of immobilized enzymes include space effect, distribution effect, diffusion effect, and so on. How to reduce the kinetics of immobilized enzyme and optimize immobilization technology is an important research direction in enzyme engineering. The use of nanotechnology to immobilize enzymes can effectively reduce the effects of spatial, distribution and diffusion effects on enzyme dynamics, thus a large recovery of enzyme activity can be obtained.

At present, there are few studies on the stabilization of thrombin. Sang, M.P. [5] evaluate the haemostatic efficacy and safety of CollaStat (Dalim Tissen Co. Ltd., Korea), a novel thrombin-containing collagen-based topical haemostatic agent used in spinal surgery. The haemostatic efficacy and safety of CollaStat were found to be non-inferior to those of Floseal since the higher limit (11.09%) of the confidence interval (CI). Li, P. [6] uses chitosan to replace carboxymethyl a degree of 65% as the carrier of thrombin and glutaraldehyde as the crosslinking agent. The results showed that the reclaim rate of immobilized thrombin was 87% when 1000 IU thrombin was immobilized using methyl chitosan with 0.1% amyl two aldehydes. The stability of the environmental adaptation is obviously improved. Lang, Y.Y. [7] used sodium alginate and chitosan as the carrier to prepare the blank microspheres by the emulsifying crosslinking method. The effect of immobilized conditions on immobilized enzyme activity was investigated.

The conditions of immobilization have been determined. Results compared with thrombin free enzyme, the stability of immobilized thrombin increased significantly and the storage time at room temperature increased significantly. The immobilized thrombin microspheres have good stability and convenient storage. In the work of Shi, P.Z. [8], an easy-to-make, rapid hemostatic, tannic acid doped calcium carbonate microsphere with a low concentration of thrombin (Thr@TA-$CaCO_3$) was developed. It is a promising hemostatic agent and can be applied in more extensive clinical applications. Giuseppe, E.U. [9] sprayed a gelatin-thrombin matrix sealant over the breach of the aneurysm, and cottonoids were gently pressed with a selfretaining spatula to stop the hemorrhage. The postoperative course was uneventful, and the patient was discharged on postoperative day 10. In the work of Liu, W. [10], a starch- polyethylene glycol sponge (St-PEG sponge) incorporated with electrospraying microspheres made from carboxymethyl chitosan and sodium alginate (CMC/SA eMPs) is employed to fabricate the topical hemostatic agent for application. To load thrombin safely and effectively, CMC/SA eMPs encapsulating thrombin compounds (eMPs@Thr) are further incorporated with St-PEG sponge to obtain eMPs@Thr/sponge. The results show that eMPs@Thr/sponge could obviously reduce blood loss and shorten the hemostasis time compared with commercial hemostatic products Kuai Kang.

A number of short peptides and proteins spontaneously assemble to form conformational stable molecular aggregates or supramolecular materials by intermolecular non covalent bonds, which have potential applications in the biomedical field. RDAD16 is a typical representative of short peptide materials, which can be applied in the biomedical field [11]-[20], such as cell culture, tissue engineering, drug delivery, and so on. The RDAD16 can self assemble into nanofibers in an aqueous solution, which can trigger the formation of gels under the action of salt.

The aim of this study is to explore the feasibility of using chemical thrombin to crosslink RDAD peptide as a fast hemostasis material. The effects of RDAD peptide concentration, calcium ion, and thrombin concentration on thrombin activity were investigated. Studies have shown that RDAD peptide can be used as an effective carrier for thrombin and widen the environmental stability of thrombin.

2. Instruments and Drugs

The RADA16 peptide (AcN-RADARADARADARADA-CNH_2, theoretical mass = 1713 g/mol, 99.5% purified powder) was synthesized commercially using solid phase synthesis methods by the Shanghai Bootech BioScience & Technology Co., Ltd., Shanghai, China. Peptide stock solutions were prepared by dissolving the peptide powders in Milli-Q water (18.2 MΩ) to a concentration of 5 mg/mL, mixing, sonicating for 30 s, filtering, and then storing at 4˚C. Thrombin was provided by Fujian Huacan Pharmaceutical Co., Ltd, which was diluted to the required concentration.

2.1. Thrombin Embedded with Different Concentrations of Polypeptide RDAD16

Polypeptide RDAD16 was dissolved in deionized water, and the concentration is set at 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml. Then 200 u thrombin was added to the polypeptide solution. The composite solution is stored in a refrigerator at 2˚C - 8˚C.

2.2. LC/MS Analysis

Each sample was analyzed by nanospray LC/MS using an LC-Packings HPLC instrument coupled to an LTQ-Velos-Orbitrap mass spectrometer. The LC gradient was 2% - 45% acetonitrile in 0.1% formic acid in 27 min at a flow rate of 250 nL/min. MS spectra were acquired in orbitrap over the range m/z 300 - 2000 with a resolution of 60,000. The top 20 most intense precursor ions were subjected to LC/MS analysis in an ion trap with dynamic exclusion set to repeat each LC/MS analysis twice within 30 s and to exclude the same mass for 60 s.

2.3. AFM for Morphology

The morphology of peptide scaffolds was analyzed using atomic force microscopy (AFM). In brief, 10 μl of 1.0% (w/v) peptide solution (Ph = 3) was placed on the surface of the clean-cut silicon wafer. Each sample was set on the mica for 30 min, washed with 100 μl water three times and then dried by blowing anhydrous 60 nitrogen. AFM images were collected with a Scanning Probe Microscope (DI Dimension 3100, Veeco Instruments Inc, US) using the dynamic force mode in ambient air. Mickromasch NSC15 micro-cantilevers were chosen with a tip radius of 10 nm, the spring constant of 40 N/m, and a frequency of 325 kHz. The 65 images were gathered with 256 × 256 pixel resolution. The main parameters of the AFM measurement were as follows: I gain/Pgain (integral gain/proportional gain): 0.20/0.30, Amplitude setpoint: 0.370 V, and scanning rate: 0.8 - 1.0 Hz. The AFM scan of each sample was repeated more than three times.

2.4. CD for Secondary Structure

The secondary structure of peptides was studied using circular dichroism (CD) measurements of 0.01% (w/v) peptide solutions. The data were collected on an Aviv model 202 spectrophotometer 30 (Aviv Instrument Inc., US) with a 1mm path length at room temperature.

2.5. Hemostasis *in Vitro*

The coagulation time was determined according to the following steps: 1) 6 healthy volunteers were routinely sterilized for 10 ml blood and mixed with anticoagulant in a silicification test tube. 2) with the addition of deionized water or 25 mmol $CaCl_2$ aqueous solution as a blank control, 0.45 ml whole blood was added into the silicified 5 ml test tube and 0.2 ml thrombin solution mixed with polypeptide was added to the whole blood, and the blood coagulation was

quickly mixed, and the blood coagulation time was recorded by the stopwatch.

2.6. Mice Liver Hemostasis Test *in Vivo*

The hemostatic performance was evaluated in the mice femoral artery and liver injury models. Forty-eight male mice were randomly divided into 3 groups, and each group had 5 mice. The three groups were the negative control group, the positive drug group (celox hemostatic powder) and the test substance group. After anesthesia, the mice were shaved off the hair of the surgical site, opened layer by layer, exposed to the liver, and made about 2.0 cm on the liver page with a scalpel × for a 2.0 cm "cross" wound, immediately sprinkle the medicine evenly on the wound, cover and press the wound with medical gauze. After a period of time, remove the gauze and observe the hemostasis. If the blood does not stop, continue to press again and again. Until the blood stops, record the hemostasis time, wipe the sample at the wound with gauze and observe whether there is bleeding and whether the sample falls after wiping.

2.7. *In Vivo* Wound Healing Analysis

In vivo tests on wound healing were carried out on male mice, weighing 180 - 220 g and 6 - 7 weeks old, and divided into five groups. After hair removal and anesthesia, two full-layer wounds were made on the mouse's back. Photographs were taken and the wound area was measured at a fixed time. For histological observations of wounds, the tissue of the mouse was immobilized with 4% paraformaldehyde and stained with the hematoxylin-eosin. In order to analyze the histological changes, wound tissue was stained with hematoxylin-eosin.

3. Results and Discussion

3.1. Synthesis of Polypeptide

Mass spectrometry is a kind of identification technology and plays a very important role in the identification of organic molecules. It can quickly and accurately determine the molecular weight of biological macromolecules, and make proteome studies from protein identification to advanced structure research of the interaction of various proteins. **Figure 1** is the mass spectrum of the polypeptide. The synthesized RDAD peptide can be seen by mass spectrometry. Its molecular weight is 1712. **Figure 2** is high-performance liquid chromatography of polypeptide, it can be seen that its purity is 95%.

3.2. CD Spectrum

Plane polarized light can produce a circular dichroism spectrum through optical material. The circular dichroism spectrum is the sum of a plane polarized light which can be decomposed into two equal parts of the left circular polarized light and the right circular polarized light component. If the wavelength of the incident plane polarized light is in the range of the group absorption peak of the polarizing substance, the polarizing substance has different absorption to the left

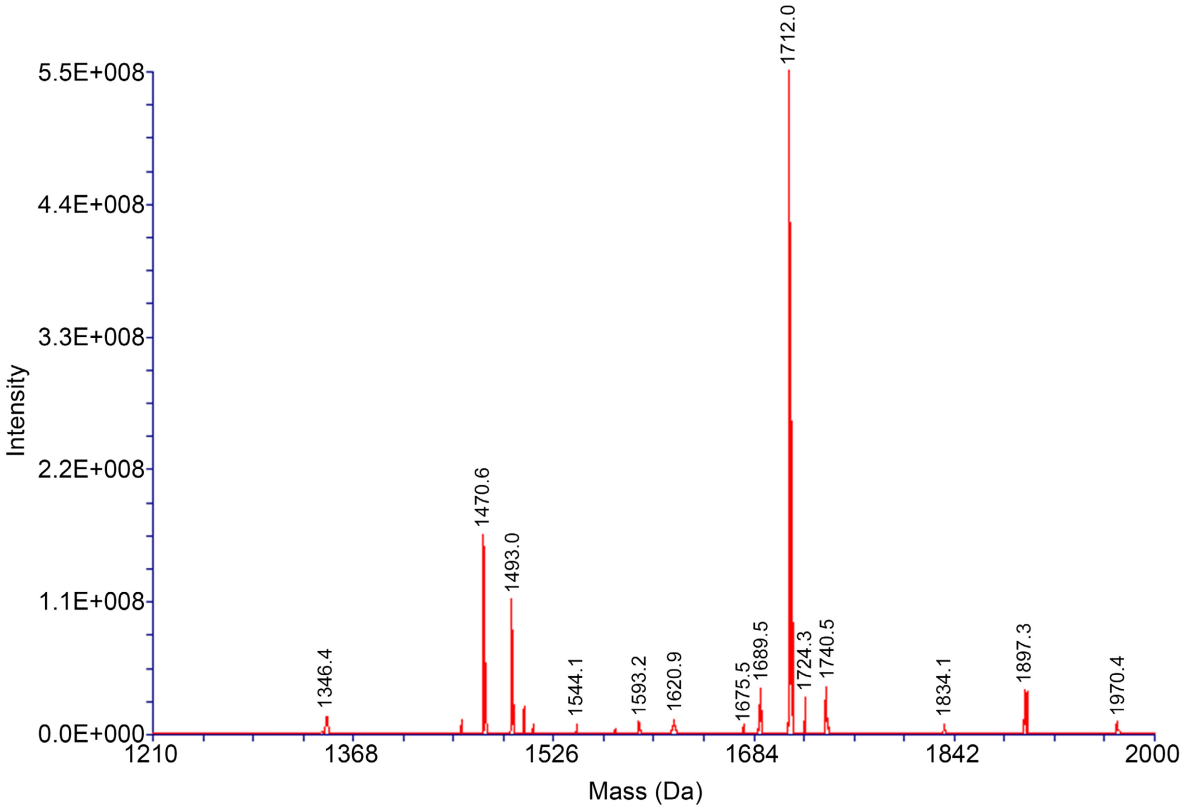
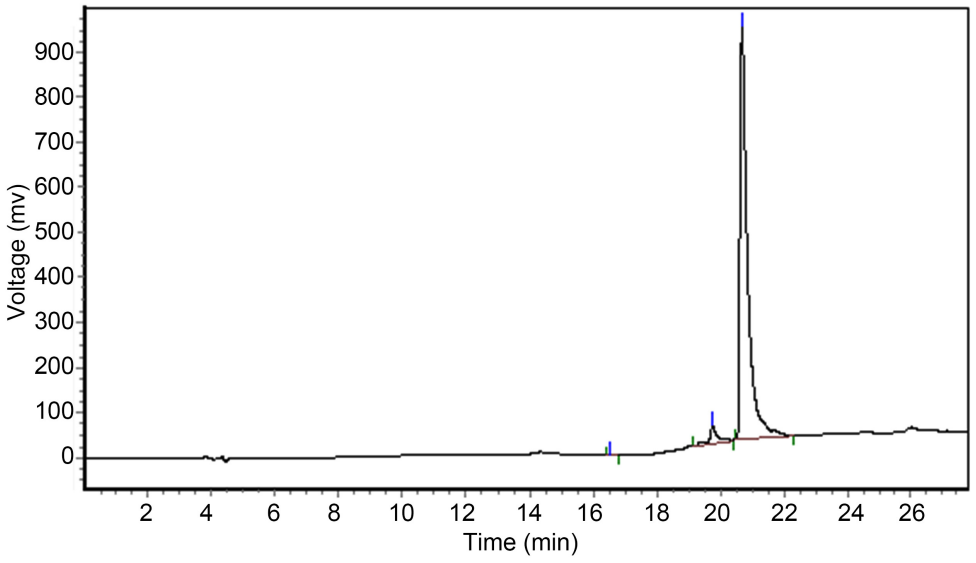


Figure 1. Mass spectrometry of RADA16.



Results

Peak No.	Peak ID	Ret Time	Height	Area	Conc.
1		16.542	1644.583	13682.736	0.0869
2		19.753	37247.859	639592.938	4.0624
3		20.683	910853.563	15091063.000	95.8507
Total			949746.004	15744338.674	100.0000

Figure 2. High performance liquid chromatography of polypeptides.

and right circular polarized light components, and ellipsoid circular polarized light appears. Circular dichroism can also be observed when the environmental asymmetry of the transition occurs. The relationship between the degree of absorption and wavelength is called Circular Dichroism (CD). CD can be used to estimate helicity in biopolymers. It is a very sensitive method for studying the conformation and conformational changes of biological macromolecules.

Therefore, CD is widely used in the determination of secondary protein structure. Some of the characteristic peaks are helpful to the identification of certain structure-specific secondary protein structure, such as Negative peaks at 202 nm and 222 nm wavelengths appear due to the α-helix, which can calculate the amount of α-helix, while the negative peaks near 192 nm and 218 nm are considered to be the contribution of β-sheet and can also be used to calculate the content of β-sheet Quantity.

In this paper, the circular dichroism of RDAD polypeptide at temperatures of 25 degrees is measured by a circular two-color spectrometer, and its secondary structure information is obtained. Figure 3 is a circular dichroism of polypeptide. The concentration of the polypeptide is 0.1 mg/ml and 1.0 mg/ml, with the typical positive peak (195 nm) and negative peak (218 nm) of the CD spectrum (Figure 3), showing a typical β-sheet structure. In an aqueous solution, 8 hydrophobic alanine side chains of stable β-sheet short peptide molecules are formed on one side, and the self-complementary ions of the positive arginine side chain and negatively charged aspartic acid side chain are on the other side. The alternate arrangement of this complementary pair of ions is very important for the formation of β-shee of tpeptide.

3.3. AFM Imaging r

Atomic Force Microscopy (AFM) is an analytical instrument for studying the surface structure of solid materials. It studies the surface structure and properties of a substance through a very weak interatomic interaction between a micro

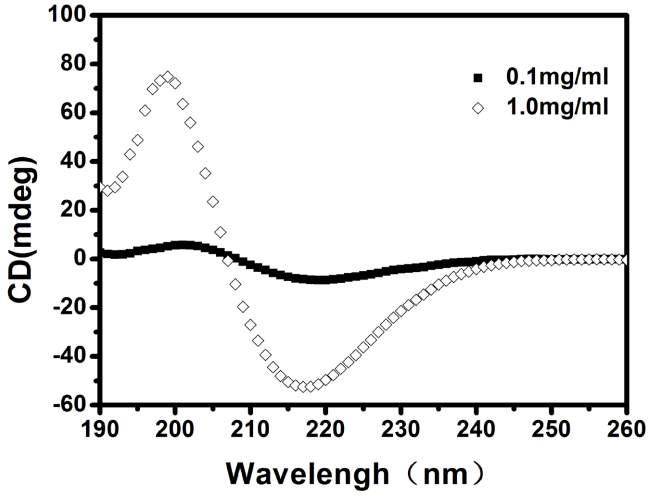


Figure 3. Circular dichroism spectrum of polypeptide compound thrombin.

force sensitive element. A microcantilever is fixed, which is extremely sensitive to the weak force, and the tiny tip of the other end is close to the sample. At this time, it will interact with the sample, and the force will change the deformation of the microcantilever or the motion state. When scanning samples, the force distribution information can be obtained by detecting these changes by sensors, thus obtaining surface structure information at nanometer resolution. The atomic mechanical microscope is composed of a microcantilever with a needle, a microcantilever motion detection device, a feedback loop monitoring its motion, a piezoelectric ceramic scanning device, a computer image acquisition, display and processing system. According to the different forms of the force between the tip and the sample, the atomic mechanical microscope can be divided into 3 working modes: tapping mode, contact mode and noncontact mode. The tapping mode is mainly used for testing soft, fragile or adhesive samples without damaging the surface of the sample. Therefore, the surface morphology of the self-assembled samples was observed by tapping mode.

Figure 4 is an atomic force microscope picture of a polypeptide, the left picture is an atomic force microscope of 0.5 mg/ml RDAD, and the right picture is atomic force microscope of Thrombin coated by RDAD. On the surface of the blank hydrogel, a striped structure was observed. In the right picture, white thrombin indicated that the thrombin was effectively entrapped by peptide.

3.4. Study on the Coagulation Time of Polypeptide Thrombin Solution *in Vitro*

Thrombin was fixed at 200 u and mixed with different concentrations of polypeptide water solution, and then measured the coagulation time of 1 h, 3 days, 5 days, 7 days, 30 days. The concentration of peptides is 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, the solvent is deionized water, and the volume is 2

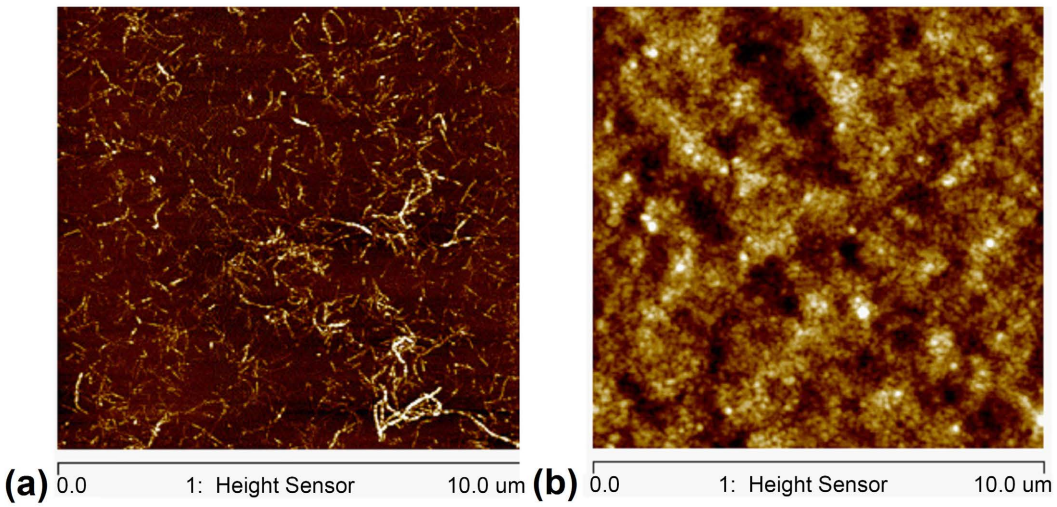


Figure 4. An atomic force microscope picture of a polypeptide, the concentration of polypeptide is 0.5 mg/ml. (a) 0.5 mg/ml polypeptide (b) 200 u thrombin aqueous solution embedded with 0.5 mg/ml polypeptide.

ml. The samples were kept in the refrigerator at 2 - 4 degrees centigrade. The coagulation time was determined according to the following steps: 1) 6 healthy volunteers were routinely sterilized for 10 ml blood and mixed with anticoagulant in a silicification test tube. 2) with the addition of deionized water or 25 mmol $CaCl_2$ aqueous solution as a blank control, 0.45 ml whole blood was added into the silicified 5 ml test tube and 0.2 ml thrombin solution mixed with polypeptide was added to the whole blood, and the blood coagulation was quickly mixed, and the blood coagulation time was recorded by the stopwatch.

Figure 5 shows a coagulant time diagram of the thrombin solution. Table 1 shows coagulation time of 200 u thrombin aqueous solution embedded with different concentrations of polypeptide after storing for 1 h, 3 d, 7 d, 30 d. Table 2 shows coagulation time of 200 u thrombin embedded with different concentration of $CaCl_2$ aqueous solution after storing for 1 h, 3 d, 7 d, 30 d. All data were expressed as mean ± standard deviation. The significance of the effect of selected parameters on the outcome variables was analyzed by multifactor analysis of variance (ANOVA). Group comparisons were made by Fisher's PLSD. Statistical

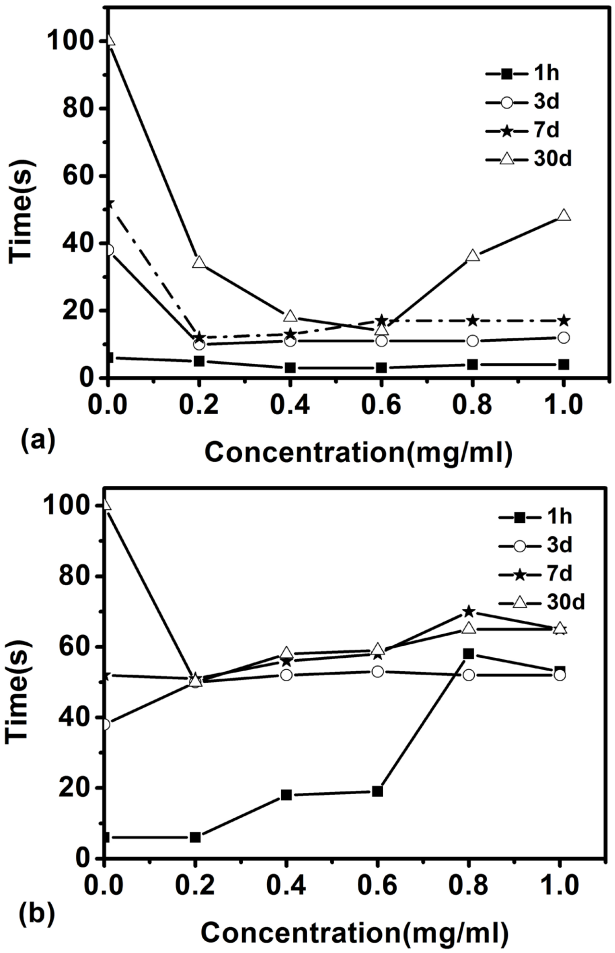


Figure 5. Coagulant time diagram of thrombin solution (a) 200 u thrombin aqueous solution embedded with different concentration of polypeptide (b) 200 u thrombin embedded with different concentration of $CaCl_2$ aqueous solution.

Table 1. Coagulation time of 200 u thrombin aqueous solution embedded with different concentration of polypeptide after storing for 1 h, 3 d, 7 d, 30 d.

Concentration	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1.0 mg/ml	Control
1 h	5 s	2 - 3 s	2 - 3 s	3 - 4 s	3 - 4 s	6 s
3 d	9 - 10 s	10 - 11 s	9 - 10 s	10 - 11 s	11 - 12 s	38 s
7 d	12 - 13 s	12 - 13 s	17 - 18 s	16 - 17 s	16 - 17 s	52 s
30 d	14 s	18 s	14 s	20 s	24 s	1 min 40 s

Table 2. Coagulation time of 200 u thrombin embedded with different concentration of $CaCl_2$ aqueous solution after storing for 1 h, 3 d, 7 d, 30 d.

Concentration	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1.0 mg/ml	Control
1 h	5 - 6 s	18 s	19 s	58 s	59 s	6 s
3 d	50 s	50 s	53 s	52 s	52 s	38 s
7 d	51 s	56 s	59 s	70 s	65 s	58 s
30 d	50 s	58 s	59 s	65 s	65 s	1 min 40 s

significance was accepted at a level of $p < 0.05$. **Figure 6** is a comparative picture of coagulation of anticoagulant blood in 30 days of a 200 u thrombin aqueous solution. The left picture is a coagulation picture of a 200 u thrombin aqueous solution embedded with 0.2 mg/ml polypeptide. The middle picture is a coagulation picture of a 200 u thrombin aqueous solution embedded with 0.4 mg/ml polypeptide. The right picture is a coagulation picture of a 200 u thrombin aqueous solution embedded with 0.6 mg/ml polypeptide. The addition of calcium ions may promote the gel formation of polypeptides, increase the space hindrance and reduce the binding capacity of the thrombin to the substrate, so coagulation time is longer than that of thrombin polypeptide solution. Therefore, after adding calcium ions, we must select the appropriate peptide quantity, which can not only guarantee the stability of thrombin, but also effectively release the thrombin factor. The peptide concentration is best at 0.4 - 0.6 mg/ml. 30 days later, the coagulation time of thrombin aqueous solution was 1 min 40 s, which was significantly different from that of thrombin polypeptide aqueous solution. Because polypeptide can release thrombin slowly, meanwhile polypeptide has the characteristics of self-assembly gelation and can form a gel network in blood, thus it can shorten the clotting time.

3.5. Evaluation of the Hemostatic Performance *in Vivo*

The liver hemostatic model is a simulation of organ type bleeding. Due to the accumulation of many static arteries in the organs, the blood flow is very rich and fragile, and the control of traumatic bleeding is very difficult. Therefore, the implementation of the liver hemostatic model can evaluate the hemostatic performance of the material authoritatively. The content of thrombin in polypeptide is 200 u, **Figure 7** shows the hemostatic model of the sample on the mice

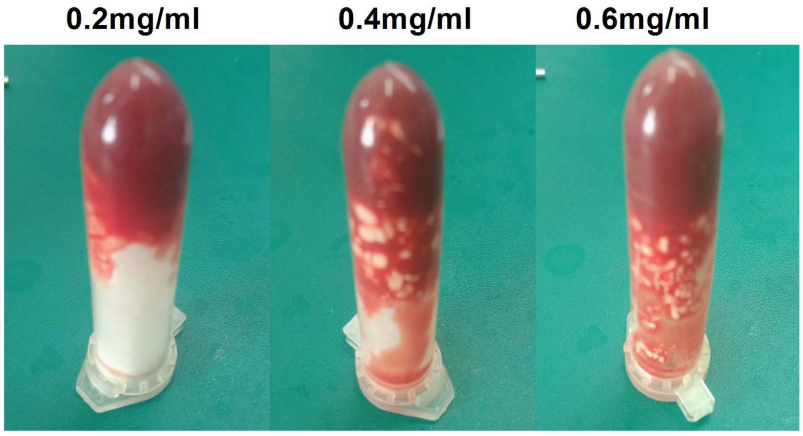


Figure 6. Comparative picture of coagulation of anticoagulant blood in 30 days of embedded with different concentration of polypeptide.

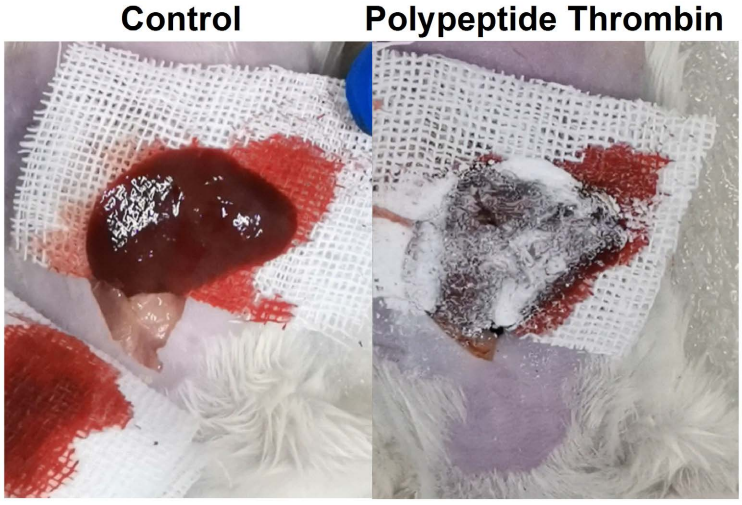


Figure 7. Hemostatic effect of hemostatic material on mice liver.

liver. It can be seen from **Figure 7** that the large blood flow caused by the wound has been completely stopped. After the hemostasis of the sample is completed, a blood scab is formed and firmly adhered to the wound.

3.6. Wound Closure Test in Mice

The establishment of the mice skin wound infection model is the key to study the healing of skin wound infection. Firstly, the mice skin wound infection model was successfully established, and the follow-up study was carried out on this basis. Hematoxylin eosin (he) staining is a method of staining nuclei and fibers with hematoxylin and eosin. As can be seen from **Figure 8**, after 6 days of infection treatment, the skin of the normal saline group was incomplete, and the collagen fibers and blood vessels of the skin of the positive control group and polypeptide thrombin group increased significantly, indicating that the wound had begun to heal. It shows that the wounds of other groups in the saline group have begun to heal, because this should be the proliferative stage of wound healing. In

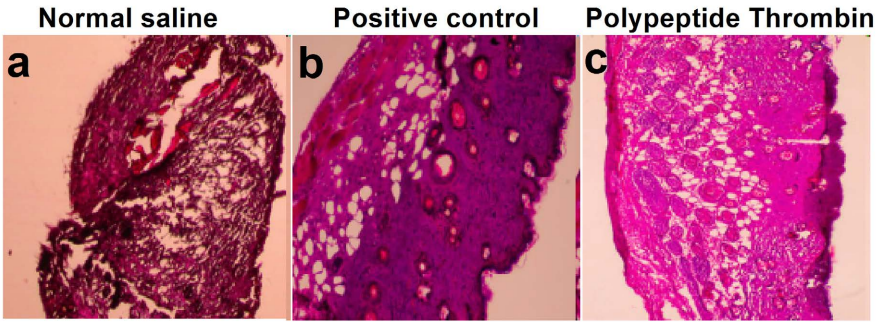


Figure 8. HE staining of mice skin in 3 group (a) Normal saline, (b) Positive control, (c) polypeptide thrombin

the proliferative stage of wound healing, skin and vascular cells begin to proliferate and form a large number of microvessels. Microvessels transport nutrients and oxygen to the wound to accelerate wound healing. Thrombin embedded with Polypeptide RDAD16 does not affect wound closure.

4. Conclusion

The thrombin spray was prepared by using the polypeptide RDAD as a stabilizer. The circular dichroism study showed that the chemical structure of the polypeptide did not change as a stabilizer. The atomic force microscope analysis showed that RDAD formed a fibrous form in the aqueous solution and could effectively encapsulate the thrombin. The simple coagulation experiment showed that the polypeptide RDAD could be the effective stability of the thrombin. The coagulant effect after 30 days showed a significant difference compared with the blank control sample.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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