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Research on PEGylation of porcine prothrombin for improving biostability and reducing animal immunogenicity

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ABSTRACT

Prothrombin is a vitamin K-dependent serine protease and plays pivotal roles in both procoagulant and anticoagulant pathway of hemostasis. In this study, prothrombin purified from porcine plasma was modified through PEGylation at N-terminal residue using 40 kDa PEG-phenyl-isothiocyanate (PIT-PEG40K). The monoPEGylated prothrombin enhanced biostability and remarkably prolonged circulating half-life in mice as compared with that of the nonmodified prothrombin. Moreover, the immunogenicity of PEGylated prothrombin in mice is significantly decreased compared to nonmodified prothrombin. These studies demonstrated the feasibility of PEGylating prothrombin as a promising way for the development of new prothrombin drugs.

Thrombin (EC 3.4.21.5) is a principal enzyme in the coagulation mechanism, in which it has multiple enzymatic and regulatory functions.[1] On one hand, thrombin may stimulate platelets to form a platelet plug and the cleavage of fibrinogen to form the insoluble fibrin clot, both of which prevent excessive loss of blood from injured blood vessels. On the other hand, thrombin activates protein C and protein S to initiate the inhibition of the coagulation process.[2] In addition to its primary roles in coagulation, thrombin has a variety of important effects on a number of cell lines upon binding to its receptors.[3,4] Prothrombin is a serine protease zymogen that is made primarily in the liver and secreted into the blood. Porcine prothrombin is synthesized as a glycoprotein consisting of a single polypeptide chain of 623 amino acid.[5] During hemostasis, prothrombin is converted to thrombin, in the final stages of the coagulation cascade by the enzyme, factor Xa. In this reaction, substrate (prothrombin), enzyme (factor Xa), and cofactor (factor Va) are complexed by virtue of mutual adherence to phospholipid via calcium bridges. Upon activation of the factor Xa, prothrombin undergoes proteolytic cleavage to release the 'pro' portion of the molecule and the two-chain thrombin molecule.[6] In addition, the thrombin produced upon activation can cleave prothrombin at one or two sites.[7] The result of the two factor Xa cleavages and the thrombin cleavage(s) is that many activation components of prothrombin are produced.[3]

Prothrombin deficiency is an autosomal recessive coagulation disorder resulting from mutations in the prothrombin gene and can cause two types of congenital disorders, hypoprothrombinemia and dysprothrombinemia, that result in excessive bleeding.[8–10] Prothrombin antigen levels in plasma decrease significantly in hypoprothrombinemia, whereas in dysprothrombinemia normal prothrombin antigen levels are detectable.[11] The major obstacles to clinical application of prothrombin in treatment of these disorders, however, lie in its short circulating half-life and high immunogenicity.[12] For this reason, the development of a methodology to modify prothrombin, which would diminish its immunogenicity while preserving biological activity, making it more suitable for therapeutic purposes has been investigated.

Covalent conjugation of polyethylene glycol to therapeutic proteins (PEGylation) increases their stability by protecting the protein from degradation, masking its immunogenic sites and reducing clearance.[13–15] Up to now, none of studies on PEGylation of the prothrombin has been published. Thus, to obtain site-specific monoPEGylated prothrombin, we attempted to extend prothrombin action through addition of a PEG chain to the N terminus of prothrombin. This site was chosen as it is very distant from the C terminus of prothrombin which is most critical for intact biological activity.[3] Furthermore, the N terminus of prothrombin is removed by factor Xa during activation of prothrombin. The active product of PEG-prothrombin is same as the native thrombin, which is strictly regulated in the blood.

Initially, the prothrombin was purified from porcine plasma by sequential barium chloride precipitation, ammonium sulfate fractionation, DEAE-cellulose and Sephadex G-100 gel filtration chromatography. About 198 mg of prothrombin was purified from 5000 mL porcine plasma with a yield of 11.6% (Table 1). Anti-prothrombin antibody was prepared in rabbit by using purified prothrombin as a soluble antigen. Western blotting analysis showed that anti-prothrombin antibody raised in rabbit could recognize porcine prothrombin with high specificities[16](Fig. 1).

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Table 1
Purification of prothrombin from porcine plasma

Purification	Volume (ml)	Protein (mg/ml)	Specific activity(U/mg)	Total activity(U)	Yield (%)
Plasma	5000	74.7	1.85	691,000	100
50–70% $(NH_4)SO_4$	100	36.0	128	460,600	66.7
DEAE-sephadex A-50	60	13.1	263.1	206,800	30
Sephadex G-100	30	6.6	404.5	80,100	11.6

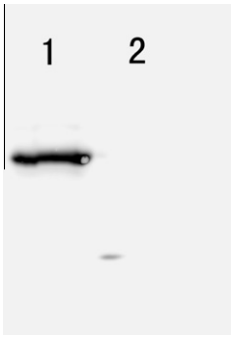

Figure 1. Western blotting analysis of the polyclonal antibody against porcine prothrombin; lane 1: serum from the mice (immunized with porcine prothrombin); lane 2: serum from the control mice (immunized with PBS alone).

According to the typical procedure[17], We further modified purified porcine prothrombin by PEGylation at the N-terminal residue using 40 kDa PEG-phenyl-isothiocyanate. Phenyl-isothiocyanate is a well-known reagent for carrying out Edman degradation and amino acid analyses by HPLC.[18] It shows a high selective reactivity with the N-terminal amines in proteins.[19]

SDS–PAGE analysis of all samples obtained in statistic orthogonal tests of PEGylation of prothrombin showed a variety of modification levels (data not shown). The highest yield of PEGylation of prothrombin was obtained by incubating 10-fold molar ratio excess of 40 kDa PIT-PEG to prothrombin in a sodium acetate solution, pH 6.2 at 25 °C for 2 h. Under this condition, the modified prothrombin was up to 39.8% in total polypeptide input.

PEGylation products were analyzed by reversed phase HPLC.[19] Two chromatographic peaks were observed (Fig. 2A), indicating the homogeneity of the fraction. These two fractions were collected and analyzed by SDS–PAGE. The analysis demonstrated that there were two distinct molecular weight bands (Fig. 2B). The band with higher molecular weight (lane 3) represented PEG-prothrombin, and the band with lower molecular weight was nonmodified prothrombin (lane 2). The longer retention time of PEG-prothrombin on reversed phase HPLC suggests the increased hydrophobicity of the PEG-prothrombin due to the PEGylation. The homogenous product implies that certain amino acid residue in prothrombin was modified by PEG, and it is very likely the N-terminal residue since a strategy that favors α-amino acid residue modification by PEG was applied.

The location of the conjugated PEG moiety was determined by a combination of analytical methods including peptide mapping, sequencing and mass spectrometry, as described.[20] As shown in Figure 3, when compared with the chromatographic profile of the digested prothrombin, PEG-prothrombin digest had a greatly diminished peak which was confirmed by mass spectrometry as the N-terminal peptide consisting residues 1–9 (ansgffeem).

Using one stage clotting assay[21], the specific bioactivity of the PEG-prothrombin (368.5 U/mg) was found to be a little less than that of the native prothrombin (404.5 U/mg). By the Line weaver–Burk plotting, the K_M for the native prothrombin was of 6.1×10^{-6} M, similar to the K_M described for PEG-prothrombin that have a K_M of 5.9×10^{-6} M. The PEG-prothrombin exhibited bioactivity toward fibrinogen that was comparable to native prothrombin. So PEGylation with a 40 kDa PEG targeted at a single site on the N-terminal amine had no deleterious effect on its specific activity in a coagulation assay.

One of the reasons for the long-term stability of PEGylated proteins circulating in vivo might be their resistance to proteinase-induced degradation.[20,22,23] To verify that prothrombin had enhanced resistance to proteolysis, we carried out an in vitro experiment in which both PEGylated and nonPEGylated prothrombin were incubated with trypsin or chymotrypsin at concentration of 1 mM for indicated times. Protein integrity was examined by SDS–PAGE. The bands representing PEGylated and nonPEGylated

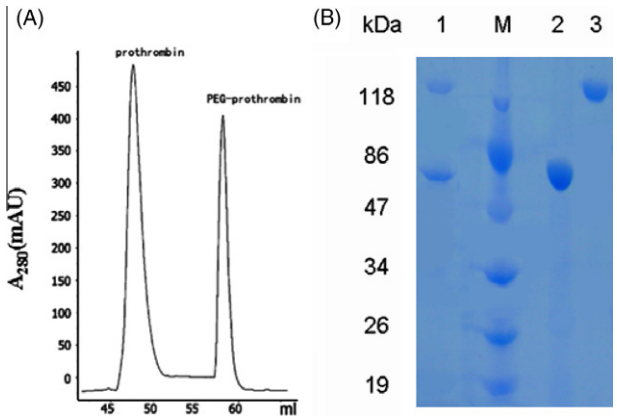

Figure 2. RP–HPLC(A) and SDS–PAGE(B) analyses of PEGylation of prothrombin; lane m, molecular weight standards; lane 1: reaction mixture; lane 2: peak I of HPLC fraction; lane 3: peak II of HPLC fraction.

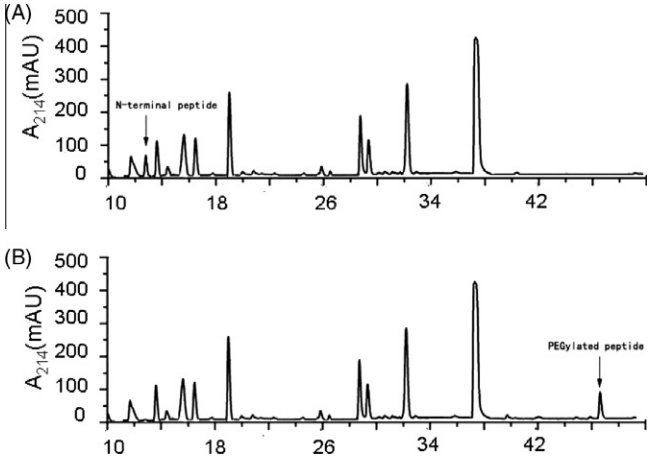

Figure 3. Comparison of CNBr-generated peptide maps of prothrombin (A) and PEG-prothrombin (B). Arrow indicated peak present in the unmodified protein, but missing in the PEGylated counterpart, was identified by MS as the N-terminal peptide.

prothrombin were quantified by densitometry scanning. The band density of nonproteases-treated prothrombin or PEGylated prothrombin is considered as 100% (indicated by control), while the band density of hydrolyzed PEGylated or native prothrombin is presented as relative percentage to the control. Figure 4 showed that the PEGylated prothrombin was more resistant to trypsin proteolysis than prothrombin. After 40 min of incubation with trypsin, 75.6% of PEGylated prothrombin still retained intact, whereas 73% of the native prothrombin was digested. When PEG-prothrombin and prothrombin were incubated with chymotrypsin, the results showed that the half-life of PEG-prothrombin was about 80 min while that of prothrombin was about 20 min (Fig. 5). These results demonstrated that the PEGylated prothrombin significantly increased resistance to proteolysis of prothrombin. This discovery was anticipated, as it was demonstrated previously that PEG could yield a protection against the approaching and attack by circulating proteases or cells.[23]

To obtain the effects of temperature on prothrombin and PEG-prothrombin, the PEG-prothrombin or prothrombin was incubated at 0, 25, 37, 42, 50, 60 °C, respectively. After 12 h of incubation, all samples were centrifuged at 15,000 rpm for 10 min. The absorbance of supernatant was measured at 280 nm to determine the remaining protein. Results showed porcine prothrombin was stable from 0 to 37 °C just like PEG-prothrombin. However when the temperature was increased to 50 °C, there was only 20% prothrombin remaining while almost 75% PEG-prothrombin remaining. When the denaturalization ratio of prothrombin was 50%, the temperature was about 46 °C, whereas the corresponding temperature of PEG-prothrombin was about 60 °C. Figure 6 showed that the PEG-prothrombin conjugates were obviously more stable against thermal denaturation. This could be related to a mechanism reported by other investigators, which suggested that PEGylation could induce a blocking of the intermolecular interactions that were involved in thermal instabilities.[24,25]

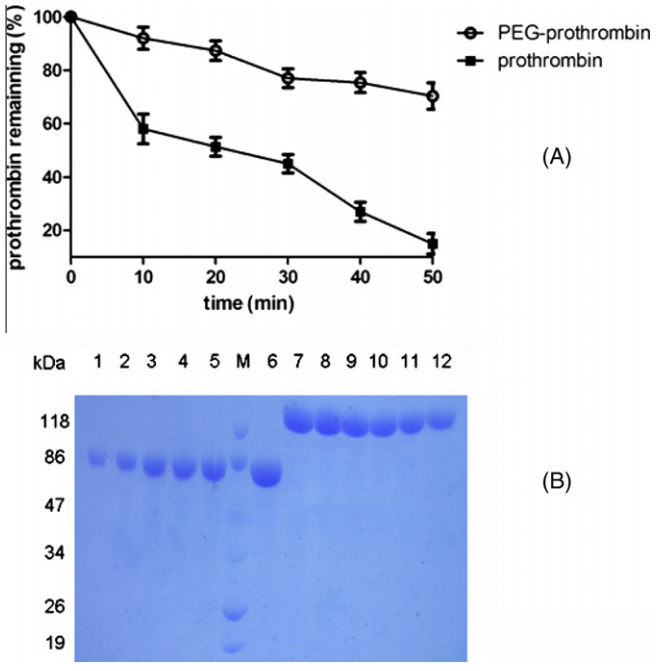

Figure 4. The relative resistance of PEG-prothrombin and prothrombin to trypsin proteolysis(A). Each data value represents means ± SD (n = 3). 500 μL of PEG-prothrombin or prothrombin (1.25 mg/mL, pH 7.4 phosphate buffer (PBS)) was incubated with trypsin at final concentration of 1 mM for indicated times. Reactions were carried out at 37 °C. The PEG-prothrombin conjugates displayed stronger resistance to typsin digestion than native prothrombin. SDS–PAGE(B) analysis of PEGylated and nonPEGylated prothrombin incubated with trypsin for indicated times. Lanes 1–6 indicate prothrombin incubated for 120, 80, 60, 40, 20, 0 min; lanes 7–12 indicate PEG-prothrombin incubated for 0, 20, 40, 60, 80, 120 min.

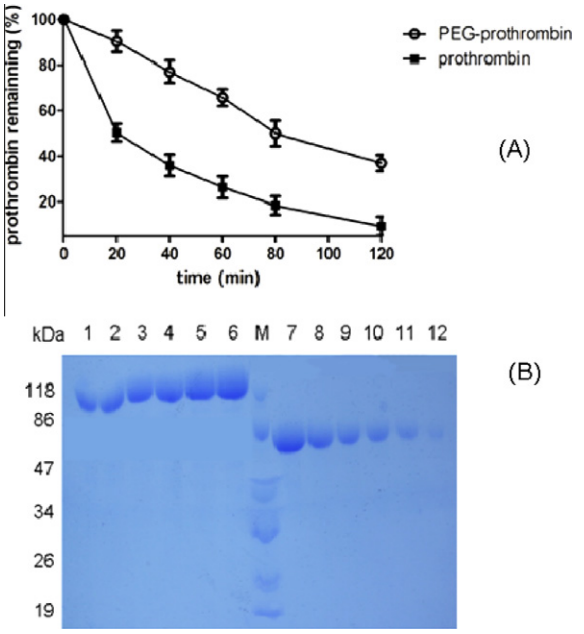

Figure 5. The relative resistance of PEG-prothrombin and prothrombin to chymotrypsin proteolysis(A). Each data value represents means ± SD (n = 3). 500 μL of PEG-prothrombin or prothrombin (1.25 mg/mL, pH 7.4 phosphate buffer (PBS)) was incubated with chymotrypsin at concentration of 1 mM for indicated times. Reactions were carried out at 37 °C. The PEG-prothrombin conjugates displayed stronger resistance to chymotrypsin digestion than native prothrombin. SDS–PAGE(B) analysis of PEGylated and nonPEGylated prothrombin incubated with chymotrypsin for indicated times. Lanes 1–6 indicate PEG-prothrombin incubated with chymotrypsin for 120, 80, 60, 40, 20, 0 min; lanes 7–12 indicate prothrombin incubated with chymotrypsin for 0, 20, 40, 60, 80, 120 min.

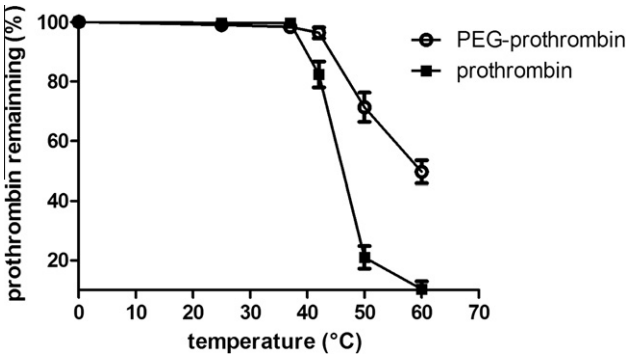

Figure 6. The effect of PEGylation on the relative susceptibility of PEG-prothrombin and prothrombin to temperature. Each data value represents means ± SD (n = 3). Temperature of PEG-prothrombin (1.25 mg/mL, pH 7.4 PBS, 500 μL) or prothrombin (1.25 mg/mL, pH 7.4 PBS, 500 μL) was incubated at 0, 25, 37, 42, 50, 60 °C, respectively. After 12 h of incubation, all samples were centrifuged at 15,000 rpm for 10 min. The absorbance of supernatant was measured at 280 nm to determine the remaining protein.

Similarly, to obtain the effects of acid or alkali, the pH of prothrombin and PEG-prothrombin was adjusted to 2, 3, 4, 5, 6, 8, 9, 10 and 11. The samples were incubated at 37 °C for 12 h and collected by centrifugation. The remaining protein in supernatant was measured by UV spectrophotometry. Results showed porcine prothrombin was stable from pH 2–7 as well as PEG-prothrombin. When the pH was greater than 9, PEG-prothrombin was significantly more stable than prothrombin. Even the pH rose to 10,

PEG-prothrombin was still stable while the denaturation ratio of prothrombin reached at 79% (Fig. 7). Therefore, the N-terminus site-specific PEGylation also enhanced the acid base stability of prothrombin significantly.

PEGylation prolongs the residence time of protein drugs in the body. Circulating levels of PEGylated prothrombin and prothrombin were measured by ELISA following subcutaneous injection. The curves of mean serum concentration–time are shown in Figure 8. Plasma concentration of prothrombin peaked at 0.75 ± 0.15 h after administration and then rapidly decreased to almost undetectable levels. In contrast, maximum plasma levels of the PEGylated were not attained until about 10 h post-administration. The PEGylated prothrombin group area under curve (AUC) was also significantly increased compared with the prothrombin-treated group. Terminal half-life for the PEGylated prothrombin increased eight-fold compared with that of the unmodified one. Administration of our PEGylated conjugates greatly extended the in vivo plasma half-life and increased the plasma exposure of prothrombin, and the PEG-prothrombin displayed even better pharmacokinetic performances than prothrombin. In addition to the protective effect of PEGylation to the protein, the elongated half-life in vivo may also be correlated with the decreased rate of its renal clearance due to the increased molecular size after PEGylation.

To investigate the fibrinogen lowering activity of PEG-prothrombin, Prothrombin or PEG-prothrombin was administrated by subcutaneous injection simultaneously. The group got PBS only was used as control. Compared with the control, the blood fibrinogen level was significantly lower following administration of prothrombin or PEG-prothrombin (Fig. 9). Moreover, PEG-prothrombin was found to be significantly more effective than the native prothrombin with P value less than 0.01 (Fig. 6). The fibrinogen level of the conjugates groups reached minimal levels on day one, then gradually increased to the same level as that of the control on day five. This result may be caused by the improved stability of PEG-prothrombin over prothrombin. First, for PEG-prothrombin, the conjugated PEG chain could improve the stability of prothrombin. Second, for the native prothrombin, most of the molecules might have been degraded before they exhibited fibrinogen lowering activity. Taken two factors together, the in vivo fibrinogen lowering activity of PEG-prothrombin was not only maintained but also enhanced as compared with that of native prothrombin.

ELISA was conducted to evaluate whether PEGylation could shield the recognition of prothrombin by specific anti-prothrombin antibodies.[23] Figure 10 showed that statistically significant differences in fluorescence intensity between unprotected prothrombin and PEGylated prothrombin were observed (two-way ANOVA, P <0.01) over the entire concentration range tested, indicated a decrease in antibody recognition of the PEG-protected prothrombin. The reduction of 62.8% in antibody recognition is noticed with PEG-prothrombin from the highest concentration when compared with the same concentration of native prothrombin. This allowed us to conclude that the attachment of PEG to prothrombin inhibits the binding of antibody to the prothrombin moiety in the conjugate. This decrease in immunoreactivity may be due to the steric hindrance yielded by the long PEG chains that blocked the interaction between prothrombin and its specific antibodies. This is con-

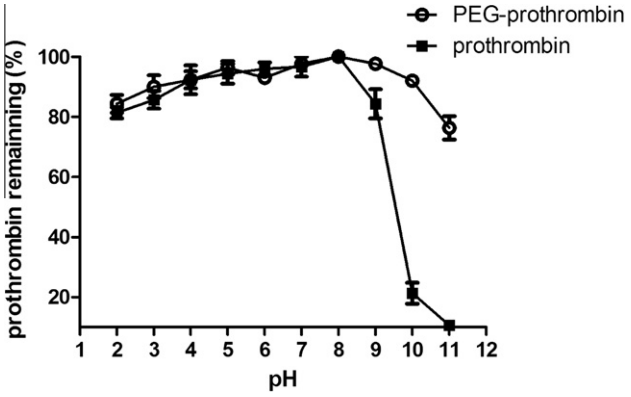

Figure 7. The effect of PEGylation on the relative susceptibility of PEG-prothrombin and prothrombin to pH. Each data value represents means ± SD (n = 3). The pH of PEG-prothrombin (1.25 mg/mL, pH 7.4 PBS, 500 μL) or prothrombin (1.25 mg/mL, pH 7.4 PBS, 500 μL) was adjusted to 2, 3, 4, 5, 6 using acetic solution and 8, 9, 10, 11 using sodium hydroxide.

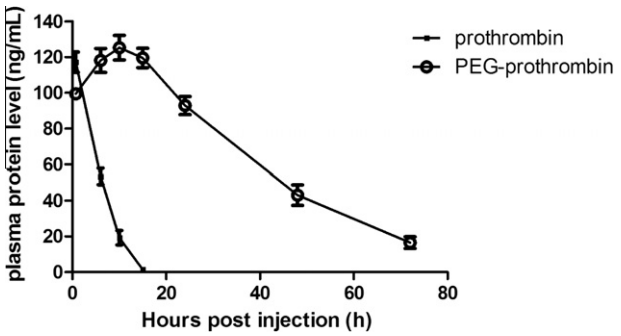

Figure 8. Mean plasma protein concentration versus time of prothrombin and PEG-prothrombin after subcutaneous injection in mice. Data were means ± SD for five mice per group. Each rat received a single 100 μg protein/kg dose. Plasma levels of the proteins were measured by ELISA.

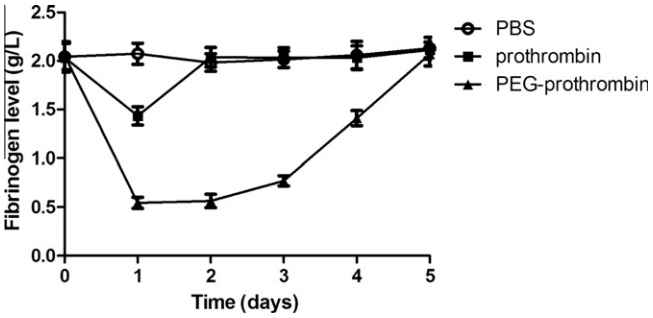

Figure 9. Biological activity assay of prothrombin. The capabilities of fibrinogen-lowering after subcutaneous administration of prothrombin or PEG-prothrombin at a dose of 50 μg/kg. Plasma levels of the fibrinogen were measured by ELISA kit, data are means ± SD for five mice per group.

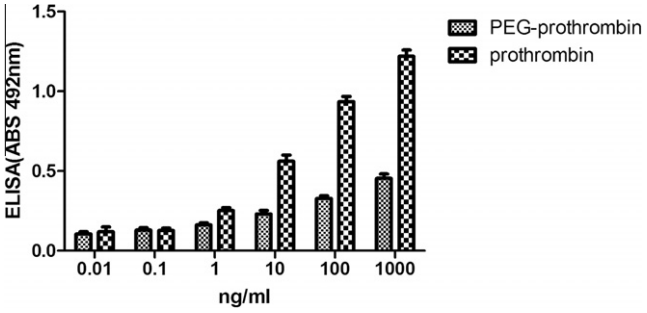

Figure 10. ELISA assay to assess antibody recognition towards prothrombin and PEG-prothrombin. Increasing amounts of either native or conjugate prothrombin species were assayed by ELISA. Statistical analysis was performed by utilizing two-way ANOVA, and results indicated that the difference was statistically significant (P <0.01).

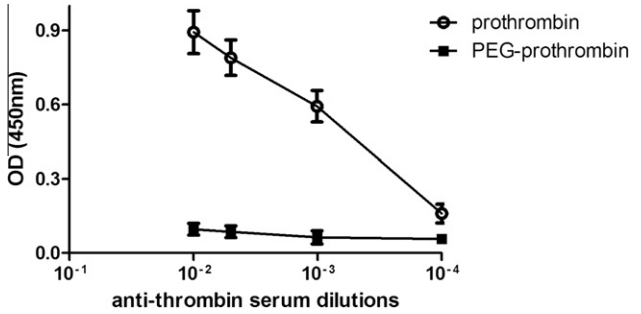

Figure 11. Immunogenicity of native and modified prothrombin in mice. The results are mean ± SD obtained with five animals/group. The solutions were prepared by dissolution of the protein in 100 μL of PBS. The solutions were intraperitonially injected, and blood samples were taken by retro-orbital bleeding at scheduled times. The blood samples were centrifuged and the antibodies were titrated by ELISA.

sistent with discovery by other investigators on other PEGylated proteins.[20,23]

To assess the immunogenicity of PEGylated prothrombin, ten BALB/c mice were divided in two groups of five animals and treated on days 1, 7, 14, 21 with 10 μg of native prothrombin (group 1), and 10 μg of PEG-prothrombin (group 2), simulating a chronical treatment (without adjuvants) with the native protein or its derivative. Serum samples were collected on the 21st day after the first immunization, and the mouse anti-prothrombin IgG levels in serum were detected by indirect ELISA using nonmodified prothrombin as the coating antigen. While the group treated with native prothrombin showed high IgG titers, mice injected with PEGylated proteins produced no detectable amount of neutralizing antibodies. (Fig. 11) Statistical analysis between native and conjugate prothrombin confirmed this significant difference (P <0.01). This suggests sufficient reduction of the immunogenicity of this protein, a basic requirement for its use as a therapeutic agent.

In summary, PEGylation of prothrombin was successfully carried out and characterized. Both in vitro and in vivo studies provide the proof-of-concept evidence of the feasibility and utility of utilizing PEG protection against an extreme environment in vitro, protease degradation, and clearance by specific opsonizing antibodies. Furthermore, our results also showed the PEGylated prothrombin did not elicit an intense immune response in BALB/c mice. Moreover, PEGylated prothrombin might find a role in the therapy of hypoprothrombinemia and dysprothrombinemia through N-terminal site-specific PEGylation.

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