



## ORIGINAL ARTICLE

# Purification of Salmon Clotting Factors and Their Use as Tissue Sealants

Louise Z. Wang<sup>1</sup>, Jed Gorlin<sup>2</sup>, Sarah E. Michaud<sup>1</sup>, Paul A. Janmey<sup>1,3</sup>, Richard P. Goddeau<sup>3</sup>, Reet Kuuse<sup>4</sup>, Raivo Uibo<sup>4</sup>, Doug Adams<sup>5</sup> and Evelyn S. Sawyer<sup>6</sup>

<sup>1</sup>LMRC #302, Hematology Division, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, MA 02115, USA, <sup>2</sup>Memorial Blood Centers of Minnesota, University of Minnesota, 2304 Park Avenue, Minneapolis, MN 55404, USA, <sup>3</sup>1010 Vagelos Laboratories, Institute for Medicine and Engineering, University of Pennsylvania, 3340 Smith Walk, Philadelphia, PA 19104, USA, <sup>4</sup>Department of Immunology, IGMP, University of Tartu, Ravila Street 19, Tartu 51014, Estonia, <sup>5</sup>Department of Orthopaedic Surgery, University of Minnesota, Box 420 Delaware Street SE, Minneapolis, MN 55455, USA, and <sup>6</sup>Sea Run Holdings Inc., 339 River Road, Arundel, ME 04046, USA

(Received 21 July 2000 by Editor Mosesson; accepted 11 August 2000)

## Abstract

Fibrin sealant prepared from the blood of farmed Atlantic salmon (*Salmo salar*) represents a potential source of well-controlled natural material with utility in a variety of clinical settings. A potential advantage of this material is a lower probability of viral or bacterial infection that has limited general approval of fibrin glues made from human or bovine proteins. This report describes the purification of fibrinogen from salmon blood, the use of fibrin glues derived from this material to promote wound healing in rats, and the antigenic response to this material. While the low ambient temperature of these cold water fish significantly lessens the probability of infectious transmission to humans, fibrinogen and factor XIII derived from *S. salar* are activated by human thrombin at 25°C and 37°C to form clots equivalent to those formed by human fibrin. We compare the reactivity of salmon and human

fibrinogen with human and bovine thrombin and the structure and viscoelastic properties of the resulting fibrin gels over a range of pH and salt concentrations. The efficacy of salmon fibrin glues in a wound healing assay and the low antigenic response to salmon fibrinogen suggest that this material may substitute for proteins derived from mammalian sources with lower probability of infections. © 2000 Elsevier Science Ltd. All rights reserved.

*Key Words:* Fibrin glue; Fibrinogen; Thrombin; Salmonid; Surgical sealant

**F**ibrin sealants or glues are well established as effective hemostatic agents with numerous applications including cardiac, thoracic, plastic, and neurosurgery, skin grafting, repair of bony defects, and treatment of gastric ulcers [1–5]. The common feature of these applications is the need for a biodegradable tissue sealant with adhesive and mechanical properties that serve to diminish bleeding or serosal leakage or to provide additional strength to surgical anastomoses. The mechanical and adhesive properties of fibrin glues result from the structures formed by fibrinogen that assembles into polymerized fibrin after proteolytic activation by

*Abbreviations:*  $\epsilon$ ACA,  $\epsilon$ -aminocaproic acid.

*Corresponding author:* Dr. Paul A. Janmey, 1010 Vagelos Laboratories, Institute for Medicine and Engineering, University of Pennsylvania, 3340 Smith Walk, Philadelphia, PA 19104, USA. Tel.: +1-215-573-7380; fax: +1-215-573-6815. E-mail: <janmey@mail.med.upenn.edu>.

thrombin. Inactivated fibrinogen is a dimer consisting of three chains  $A\alpha$ ,  $B\beta$ , and  $\gamma$  linked covalently by disulfide bonds [6,7]. When a wound occurs, thrombin cleaves peptides from the N-termini of the  $A\alpha$  and  $B\beta$ -chains, creating fibrin monomers. The fibrin monomers align in staggered formation to form two-stranded protofibrils that thicken and branch causing a gel or fibrin clot to form [8]. In the presence of calcium, factor XIIIa adds covalent bonds to reinforce the non-covalent contacts between the fibrin monomers and other proteins, notably fibronectin, become bound to the surface of the fibrin strands. The fibrin clot traps platelets, preventing further blood loss, and forms scaffolding into which phagocytes and later fibroblasts migrate to promote wound healing.

Since the normal initial response to wounding is the formation of a blood clot stabilized by a three-dimensional fibrin network, fibrin glues derived from autologous blood plasma can serve some of these functions with a minimal chance of bacterial or viral infection. In most cases, however, autologous glues cannot be prepared in advance, so material derived from blood bank cryoprecipitate or from commercial sources utilizing bovine or human proteins has been used [9].

Recent concerns about Creutzfeld–Jacob disease linked to bovine spongiform encephalopathy (BSE) [10] as well as the possibility of transmitting viral or bacterial infections from human-derived proteins suggest that methods to improve the safety and efficacy of coagulation products need to be examined. Since the major proteins in fibrin glues, fibrinogen, thrombin, and fibronectin, have complex tertiary structures or are too large to produce as recombinant proteins by current methods, they must be purified in native form from natural sources. One approach is to purify these proteins from the blood of animals with clotting systems compatible with humans, but from which the possibility of disease transmission is minimal.

Blood products from the cold water fishes such as salmon have a lower probability of transmitting infectious agents to humans than blood from mammalian sources. This is due to both the evolutionary distances between fish and humans and the much lower body tempera-

ture, corresponding to ambient water temperature, in salmon. Moreover, since salmon blood normally clots over a wide range of temperatures, clotting factors derived from it may function more effectively than mammalian proteins under field or surgical conditions requiring lowered temperatures.

This report describes the purification of fibrinogen from salmon blood, the use of fibrin glues derived from this material to promote wound healing in rats, and the antigenic response to this material. We also compare the reactivity of salmon and human fibrinogen with human and bovine thrombin and the structure and viscoelastic properties of the resulting fibrin gels over a range of pH and salt concentration.

## 1. Materials and Methods

### 1.1. Blood Collection and Plasma Preparation

Anti-coagulated blood was prepared by drawing blood from the caudal vein of 2–5-kg salmon that had been reared in sea cages and anesthetized by immersion in oxygenated ice water. All subsequent purification steps were done at 4°C. Blood was drawn into an evacuated 15-ml glass tube containing 0.5 ml of either 40-g/l  $Na_2EDTA$  dihydrate (0.1 M) or 540-mM sodium citrate. The final concentration of 3.3-mM EDTA or 18-mM citrate was the minimum required to prevent clotting. The blood was held on ice for less than 4 h before centrifugation at  $1500 \times g$  for 20 min at 4°C. As soon as plasma was separated from the sedimented cells, plasma samples were pooled in 500-ml volumes and mixed with 5-ml 1-M benzamidine, 10-g  $CaPO_4$ , and 15-g  $\epsilon$ -aminocaproic acid ( $\epsilon$ ACA). After 30-min incubation, the insoluble  $CaPO_4$  was sedimented by centrifugation at  $5000 \times g$  for 20 min to remove surface-adsorbed coagulation factors, and 2 l of pooled supernatant was immediately applied to a 150-ml column of gelatin-Sepharose to remove fibronectin. In some cases, aprotinin was substituted for  $\epsilon$ ACA, and the supernatant was also applied to a lysine-Sepharose column to remove plasminogen. Fibrinogen levels and thrombin times in citrated plasma were measured with an Organon-Technika MDA-180 Coagulation Analyzer at

the clinical chemistry laboratory of the Boston Children's Hospital. The  $\text{CaPO}_4$ -containing pellet was used to purify salmon thrombin by a method to be described elsewhere (Michaud SE, Wang LZ, Sawyer ES, Janney PA, unpublished experiments). Reagents and chromatography resins were purchased from Sigma (St. Louis, MO) unless otherwise noted.

### 1.2. Purification of Salmon Fibrinogen

Protein fractionation procedures were done at 4°C unless otherwise indicated. Fibrinogen was prepared by two methods, employing precipitation either by ammonium sulfate or ethanol. In a modified method of Mosher and Blout [11], one-part 100% saturated ammonium sulfate in 50-mM Tris, 150-mM NaCl, pH 7.4 (T7 buffer) was added on ice to three parts of plasma to precipitate the fibrinogen for 1 h. After 15 min of centrifugation at  $17,000 \times g$ , the pellets were resuspended in 0.05-M sodium phosphate, 0.01-M sodium-EDTA, pH 6.6 (10–20 ml/100 ml of plasma) and precipitated again with 25% saturated ammonium sulfate. The pellets prepared as described above were resuspended and dialyzed against T7 buffer containing also 50-mM  $\epsilon$ ACA, pH 7.4 (T7 +  $\epsilon$ ACA).

An alternative method to purify fibrinogen is based on the method of Murtaugh et al. [12]. In this case, cold ( $-4^\circ\text{C}$ ) ethanol was added dropwise with gentle stirring to plasma at  $-4^\circ\text{C}$  to a final concentration of 13% (v/v). The precipitate was sedimented by centrifugation, resuspended in 0.05-M sodium phosphate, 0.01-M sodium-EDTA, pH 6.6, and reprecipitated with 13% ethanol. The precipitate was then dissolved in and dialyzed against T7 +  $\epsilon$ ACA.

### 1.3. Turbidity

The relative size of fibrin fiber diameters was estimated by measuring optical density (OD; turbidity) at 600 nm in 1-cm path length disposable cuvettes with a sample size of 1 ml. Each sample contained 1-mg/ml salmon fibrinogen and 6-mM  $\epsilon$ ACA in 20-mM Tris and various concentrations of NaCl,  $\text{CaCl}_2$ , and pH. When [NaCl] was varied, each solution contained 10-mM  $\text{CaCl}_2$  to ensure activation of factor XIIIa at

pH 7.4. When pH was varied, each sample contained 150-mM NaCl and 10-mM  $\text{CaCl}_2$ . When  $[\text{Ca}^{2+}]$  was varied, each sample contained 150-mM NaCl, pH 7.4. Polymerization was initiated by adding 1-u/ml human thrombin. Clots were allowed to sit overnight at room temperature before analysis. The results were graphed using Kaleidagraph software.

### 1.4. Electrophoresis and Immunoblotting

An equal volume of Tris-glycine sample buffer (120-mM Tris, 4% SDS, 20% glycerol, 0.005% bromophenol blue, and 5%  $\beta$ -mercaptoethanol, pH=6.8) was added to 2-mg/ml protein solutions and for analyses of sedimented fibrin clot, enough sample buffer to produce a 1-mg/ml solution. Fibrin clots were made by adding 0.5-U human thrombin and 10-mM  $\text{CaCl}_2$  1-mg/ml salmon fibrinogen dissolved in 50-mM Tris pH=7.4 and 150-mM NaCl. Samples were then vortexed vigorously and boiled for 5 min.

Samples were analyzed using SDS-10% PAGE. Twenty-five micrograms of protein were loaded into each lane. Proteins were visualized by Coomassie blue R-250 (BioRad, Hercules, CA) staining and by immunoblotting. Proteins transferred onto an Immobilon-NC transfer membrane (Millipore, Bedford, MA) were incubated with TTBS (153-mM NaCl, 10-mM Tris pH=7.4, and 0.1% Tween 20) and 4% non-fat dry milk (BioRad), and then probed with an  $\alpha$ -chain (Cambio, Cambridge, UK) or a  $\beta$ -chain (ARP, Belmont, MA) fibrinogen polyclonal antibodies each used at 1:5000 dilution in TTBS. HRP-conjugated secondary antibodies were used at 1:5000 dilution in TTBS and 4% non-fat dry milk. Immunoblots were developed with Kodak BioMax MR film (Fisher, Pittsburgh, PA) using an HRP-targeted chemiluminescent substrate (Pierce, Rockford, IL).

### 1.5. Rheologic Testing

Viscoelastic parameters that quantify the elastic and dissipative resistance of a material to deformations were measured using a Rheometrics RFS-II fluid spectrometer (Piscataway, NJ) by standard methods described elsewhere [13,14] and applied to clots of human fibrin and blood

plasma in previous studies [15,16]. In brief, the most important parameter is  $G'$ , the elastic or storage shear modulus defined by oscillatory deformation of a sample. The shear modulus is the ratio of the deforming stress (force per area) to the degree of deformation (quantified as strain) when the stress is applied parallel to one surface of a sample. Viscoelastic gels in general and fibrin clots in particular have elastic moduli that depend slightly on the frequency of deformation and depend strongly on the strain.

### 1.6. Shear Moduli of Salmon Fibrin Clots

Salmon fibrinogen (2.3 mg/ml) was polymerized by 0.28-u/ml bovine thrombin in 50-mM Tris, 150-mM NaCl, pH 7.4 at 23°C in the sample chamber of a Rheometrics RFS-II instrument using cone and plate geometry. Shear moduli during polymerization and gelation were measured by performing oscillatory deformation of maximal strain amplitude 2% at a frequency of 1 rad/s every 30 s after addition of thrombin. When the sample had reached steady state, after approximately 2 h or  $>20 \times$  the clotting time, the frequency dependence of the shear modulus was measured at 2% strain, and the strain dependence of the modulus measured at a frequency of 10 rad/s. Details of the rheologic instrumentation and method are described elsewhere [13].

To test the effects of repeated freeze/thaw cycles and lyophilization on salmon fibrin rheology, 0.5-ml samples of salmon fibrinogen were taken from a larger aliquot of 10-mg/ml fibrinogen in T7 buffer that had been thawed in water at room temperature. Two samples were frozen again 1 h after initial thawing. One was kept frozen at  $-80^\circ\text{C}$  before being thawed again. The other was lyophilized and reconstituted with 500- $\mu\text{l}$  water for 10 min at 23°C before measurement.

### 1.7. Force-Extension of Rat Skin Wounds After Healing with Fibrin Glues

Juvenile Wistar rats were anesthetized according to University of Minnesota Research Animal Resources guidelines including pentobarbital (40–50 mg/kg ip), hair was removed, and the skin was sterilized with blood donor-grade ster-

ilization swabs. Analgesics (1% Xylocaine or bupivacaine) were administered subcutaneously along 2.5-cm cephalocaudal lines, flanking the spine by about 1 cm. Full skin thickness incisions were made, and incisions were closed and sealed with 1 ml either human cryoprecipitate (25 mg/ml) or salmon fibrinogen (19 mg/ml). The fibrinogen was polymerized by addition of 5-U/ml bovine thrombin and 7-mM  $\text{CaCl}_2$ .

After incision, rats were housed individually and euthanized at 7 days post-operative according to the animal investigation protocol using Nembutal. Two rectangular specimens (5  $\times$  20 mm) were cut from the skin to include the incision at midsection. The strength of the incisions was measured by obtaining stress strain profiles with a MTS MicroBionix (Eden Prairie, MN) ultra-low force mechanical test system. Each specimen was clamped in custom grips allowing specimen alignment with the direction of the pull. A preload of 0.1 N was applied to establish zero extension, and then the specimen was tested by measurement of its resistance to stretching as it was elongated at a rate of 0.2 mm/s.

### 1.8. Immunologic Testing

Development of antibodies due to acute exposure to salmon fibrinogen was assessed using standard immunoelectrophoresis methods on samples of blood taken from rats and mice 36 and 120 days after application of fibrin glues to the experimental incision. Positive controls for the production of anti-fibrinogen antibodies were obtained by multiple injection of fish fibrinogen with Freund's adjuvant at multiple sites in rats followed by immunoelectrophoresis of blood taken 30 days after the last injection.

Human sera for antibody tests were obtained from the serum bank of the Department of Immunology, University of Tartu, Estonia. Altogether, 133 practically healthy persons of age 15–78 years and 39 patients with different autoimmune disorders including systemic lupus erythematosus, rheumatoid arthritis, and primary biliary cirrhosis were studied. Sera were kept in aliquots at  $-20^\circ\text{C}$  before antibody assays.

An assay for anti-salmon fibrinogen and fibronectin antibodies in human blood was made

using standard ELISA methods [17] to identify autoantibodies in serum samples from 133 normal and 39 patients with autoimmune disorders. Parallel samples (serum dilution 1:100) were measured in 96-well ELISA plates using salmon fibrinogen and salmon fibronectin as antigens (coating concentration = 1  $\mu$ g/ml). Anti-human IgG ( $\gamma$ -chain specific) alkaline phosphatase conjugate (DAKO, Denmark) was used as secondary antibody. In some experiments, anti-human IgM and IgA conjugates (DAKO) were also used.

## 2. Results

### 2.1. Purification of Salmon Fibrinogen

Fibrinogen levels in salmon blood plasma ranged from 157 to 600 mg/dl compared to a human reference range of 140–400 mg/dl. Thrombin times ranged from 4.1 to 9.4 s with a mean of 7.1 s compared to a human reference range of

12–16 s. There were no significant differences in fibrinogen levels or thrombin times in plasma taken at different water temperatures, salinity, or stages of sexual maturity.

Fibrinogen purified by ammonium sulfate precipitation was approximately 90% pure after two precipitations as measured by densitometry of bands after SDS-PAGE and by clotting tests (data not shown). The average yield was approximately 44% based on an initial average concentration of 380 mg/dl. Ethanol precipitation produced fibrinogen of similar purity and a somewhat higher yield of approximately 60%. Fig. 1A shows the electrophoretic mobility of salmon fibrinogen under reducing and non-reducing conditions. When the subunits are separated by reduction with  $\beta$ -mercaptoethanol (Lane 2), two bands appear between molecular weight markers of Mr = 52,000 and 72,000 with the lower band staining approximately twice as intense as the upper band. These two mobilities are similar to those of the B $\beta$  and  $\gamma$ -chains of human fibrino-

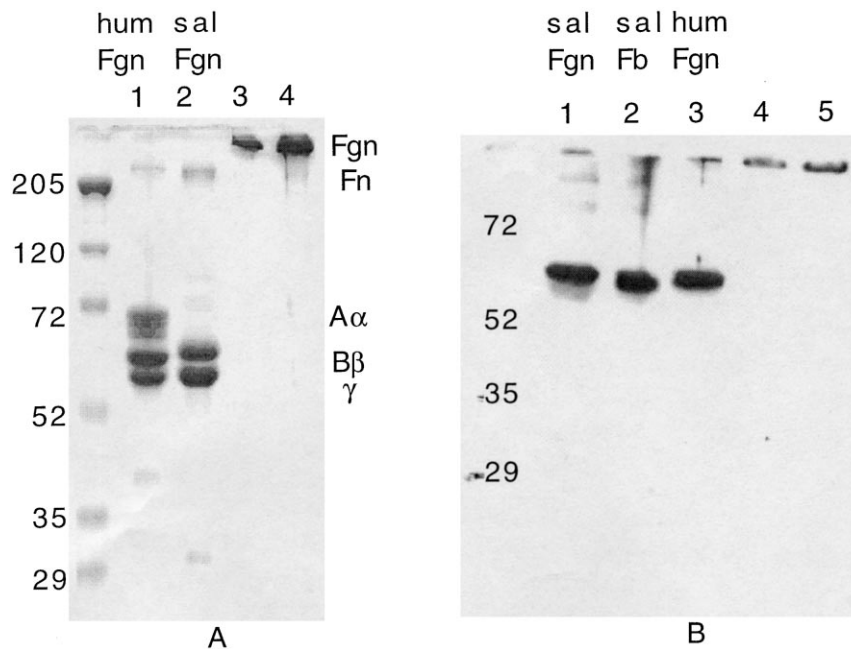


Fig. 1. SDS-PAGE analysis of reduced and unreduced salmon fibrinogen prepared using the ammonium sulfate and ethanol methods. Fig. 1A shows a Coomassie blue-stained gel with human (Lanes 1 and 3) or salmon (Lanes 2 and 4) fibrinogen run in the presence (Lanes 1 and 2) or absence (Lanes 3 and 4) of reducing agent. Molecular weight markers and corresponding molecular weights are shown on the left side, and identification of fibrinogen subunits and co-purifying fibronectin (Fn) are depicted on the right side. Fig. 1B shows an immunoblot using an antibody to the human  $\beta$ -chain added to salmon (Lanes 1 and 4) or human (Lanes 3 and 5) fibrinogen run in the presence (Lanes 1–3) or absence (Lanes 4 and 5) of reducing agent. Lane 2 shows the slight shift in mobility after treatment of salmon fibrinogen with salmon thrombin to form fibrin (Fbn).

gen (Lane 1), and no band co-migrating with the human  $A\alpha$ -chain is seen.

An immunoblot of salmon and human fibrinogens probed with an antibody to human  $\beta$ -chain (Fig. 1B) shows a reactive band at the same molecular weight for both species, suggesting that the upper band seen for salmon fibrinogen is the  $B\beta$ -chain. An antibody to the human  $\alpha$ -chain reacted with the lower band of salmon fibrinogen (data not shown). The greater mobility of this band compared to that of the  $B\beta$ -chain suggests either an anomalous electrophoretic mobility of the  $A\alpha$ -chain or a slight degradation of salmon  $A\alpha$ . No band at higher apparent molecular weight was observed even in freshly drawn plasma from blood added immediately to  $\epsilon$ ACA. Furthermore, non-reduced samples of salmon fibrinogen analyzed by both Coomassie blue (Fig. 1A) and immunostaining (Fig. 1B) consistently showed a single

band above 300 kDa, (Lane 4) showing that any possible degradation is no more than normally seen with pure preparations of human and other mammalian fibrinogens. The primary structures of salmon fibrinogen subunits are not yet known, and the true molecular weights of the salmon fibrinogen subunits or their extent of glycosylation may therefore not be identical to those of mammalian fibrinogens.

Addition of either human or salmon thrombin to ethanol-purified fibrinogen produced clots that contained fibrin subunits with the slightly altered electrophoretic mobility expected when fibrinopeptides are cleaved from the  $A\alpha$  and  $B\beta$ -chains. Fig. 2 shows the change in mobility of the fibrinogen chains after clotting in the presence and absence of 10-mM  $Ca^{2+}$  required for the activation of endogenous factor XIII that normally copurifies with fibrinogen unless steps are taken to remove it. A comparison of Lanes 1 and 2 or 4 and 5 shows that one of the two fibrinogen chains that overlap at an apparent molecular weight of 48 kDa disappears, and a band corresponding to a polypeptide of  $Mr = 80,000$  appears after addition of either human or salmon thrombin in the presence but not the absence of 10-mM  $Ca^{2+}$ . This altered migration presumably represents transglutamination of the  $\gamma$ -chain to form  $\gamma$ - $\gamma$  dimers. The altered mobility of the  $B\beta$ -chain after reaction with thrombin is also seen in the immunoblot shown in Fig. 1B.

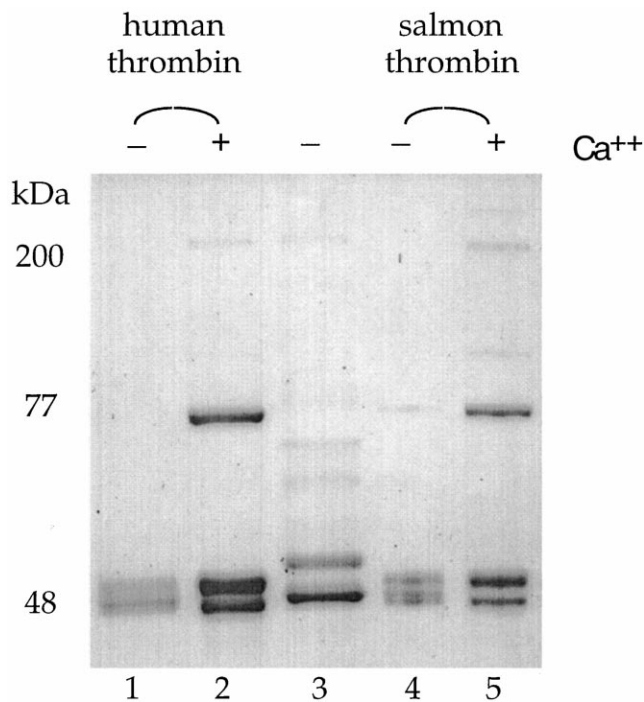


Fig. 2. SDS-PAGE analysis of salmon fibrinogen before and after cleavage by thrombin. Ethanol purified salmon fibrinogen before (Lane 3) and after addition of human (Lanes 1 and 2) or salmon thrombin (Lanes 4 and 5) in solution containing no added  $Ca^{2+}$  (Lanes 1 and 4) or 10-mM  $Ca^{2+}$  (Lanes 2 and 5). Molecular weights from co-migrating marker proteins are shown on the left. Putative identifications of fibrinogen subunit chains are indicated on the right.

## 2.2. *In Vitro* Rheologic Characterization

When the viscoelastic resistance to shear deformation is measured during polymerization of salmon fibrin, the elastic storage shear modulus  $G'$  shown in Fig. 3A shows a steep rise after the clotting time followed by a leveling off to a value similar to that of human fibrin [16] and much higher than the loss modulus  $G''$ . The values of  $G'$  and  $G''$  are approximately independent of the frequency of deformation over a large range (Fig. 3B) but are strongly dependent on the maximal strain of the measurement. Fig. 3C shows that the elastic resistance to deformation is more than 10 times as great at 35% strain as it is at 2% strain. The salmon fibrin gel also shows no detectable hysteresis after strain up to at least 37%, since subsequent measurements at decreas-

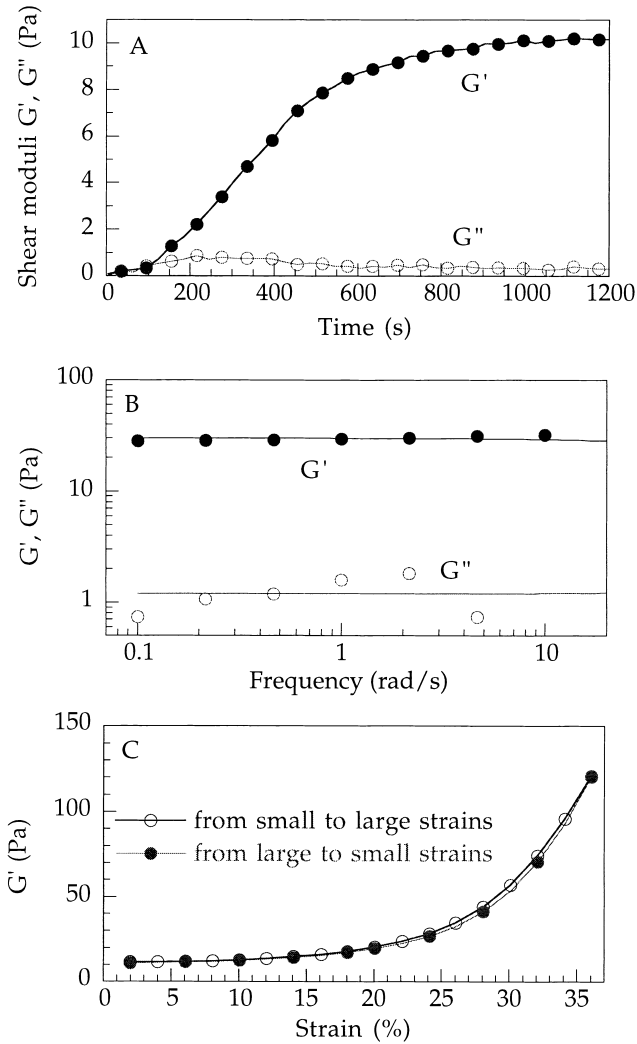


Fig. 3. (A) Development of elastic storage shear ( $G'$ , closed circles) and loss moduli ( $G''$ , open circles) during polymerization of salmon fibrin following addition of bovine thrombin (0.3 u/ml) to 2.3-mg/ml salmon fibrinogen. (B) Frequency dependence of elastic moduli of salmon fibrin gel. (C) Shear storage modulus of 2.3-mg/ml salmon fibrin measured at increasing maximal strain amplitude during oscillatory measurement at 10 rad/s.

ing strain amplitudes are identical to those performed prior to the large strain deformation.

In agreement with previous studies of mammalian fibrin, the elastic modulus of salmon fibrin is strongly dependent on the protein concentration, and the ability of the protein to polymerize is retained after freeze-drying and reconstitution. Fig. 4 shows that the elastic modulus is more than 10 times greater for a 8-mg/ml gel compared to 2.7 mg/ml (Fig. 3) even

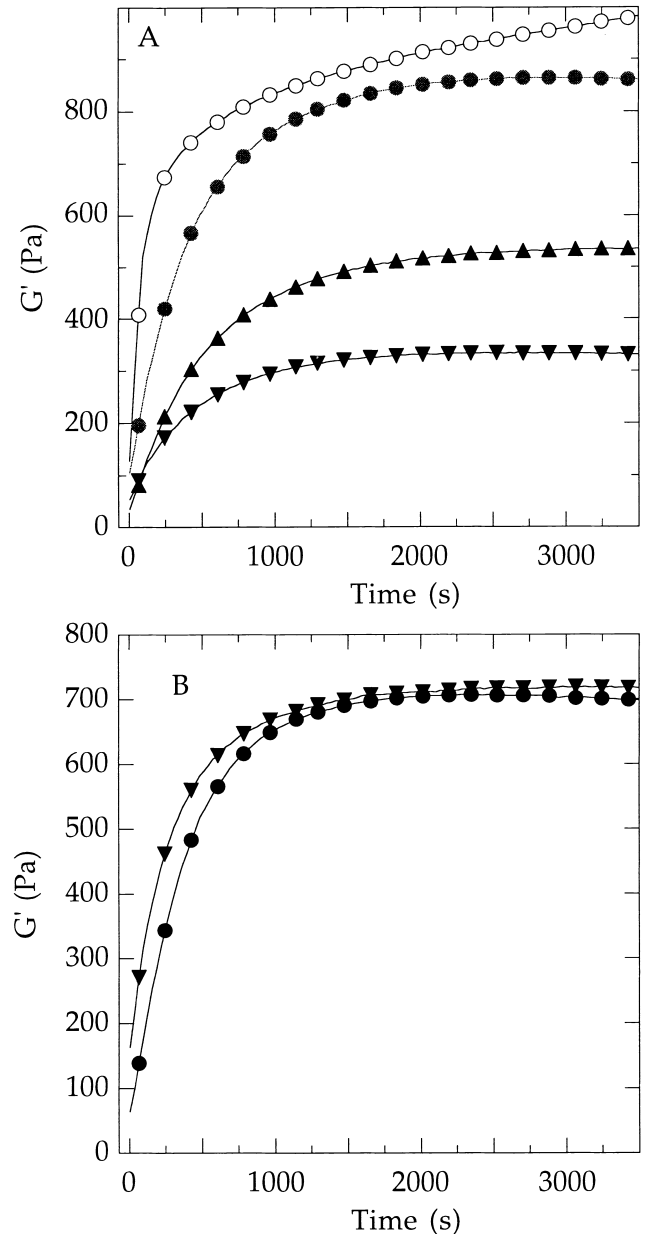


Fig. 4. Increase in elastic modulus during polymerization of salmon fibrinogen after freeze/thaw cycles and lyophilization. Salmon fibrinogen (8 mg/ml) was polymerized with 1-u/ml salmon thrombin in T7+ $\epsilon$ ACA (closed symbols) or T7+ $\epsilon$ ACA+3-mM CaCl<sub>2</sub> (open symbols). Fibrinogen was prepared by thawing an aliquot that had been frozen immediately after the final centrifugation step of the purification (circles), frozen and thawed twice (triangles down), or frozen twice, lyophilized, and reconstituted with water (triangles up). Fibrinogen was prepared from plasma depleted of fibronectin (A) or plasminogen (B: circles, frozen/thawed; triangles/lyophilized).

accounting for the greater strain hardening of the lower concentration gel. Fig. 4A also shows that the rise of the gel elasticity is slightly faster and the final extent greater in 3-mM  $\text{Ca}^{2+}$  consistent with a contribution of  $\text{Ca}^{2+}$  both to the structure of the fibrin fibers and the activation of factor XIII. Fig. 4A and B also show that a sample that had been lyophilized from a once-thawed aliquot retained much of the clot strength of the once-frozen sample. Fibrinogen prepared from plasma passed over a lysine-Sepharose column to remove plasminogen was especially well preserved after lyophilization (Fig. 4B).

### 2.3. Turbidity Analysis

Measurement of the OD of the fibrin clot samples reflects the thickness of the fibers in the clot. Fig. 5 shows the relationship between pH or [NaCl] and OD of the fibrin clots. Samples were made using either human or salmon fibrinogen clotted with salmon thrombin.

Fig. 5A shows the turbidity of human and salmon fibrin clots through a pH range of 6.0–9.0. At pH 6.0, human and salmon fibrin both had an OD of 0.6. With an increase in pH, the human fibrin clot gradually decreased in OD to very low levels at pH 8.0 and remained low through pH 9.0. In contrast, salmon fibrin clot turbidity showed very little dependence on pH over the range from 6 to 8 and dropped only to 0.45 at pH 8.5 and 9.0.

The effect of NaCl concentration on fibrin clot turbidity (Fig. 5B) was examined with [NaCl] from 10 to 500 mM. Human and salmon fibrin clots began at an OD reading of 0.7 at low ionic strength. The human fibrin clot showed a slight decrease in OD from 0 to 100-mM NaCl. From 100 to 150 mM, the OD dropped from 0.7 to very low levels. The OD remained low from 150 mm to 500-mM NaCl. The clots formed with salmon fibrinogen had an OD of 0.7 at 0-mM NaCl that decreased to 0.35 at 300-mM NaCl and leveled off to 0.3 at the final concentration of 500-mM NaCl. In general, the clots formed with salmon fibrinogen had a higher and more constant turbidity than the human fibrinogen clots over a broad range of both pH and [NaCl].

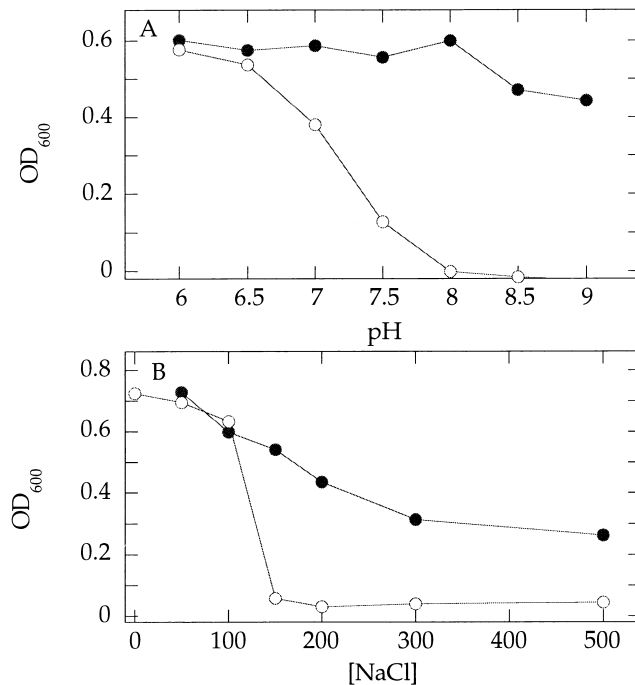


Fig. 5. (A) The effect of pH on the OD of salmon and human fibrinogen clotted with salmon thrombin. Samples consisted of 1-u salmon thrombin, 1-mg/ml salmon or human fibrinogen, 150-mM NaCl, 3-mM  $\text{CaCl}_2$ , 6-mM  $\epsilon\text{ACA}$ , and 20-mM Tris, varying pH. Closed circles represent fibrin clots made with salmon fibrinogen, and open circles represent fibrin clots made with human fibrinogen. Measurements are based on the mean absorption value of three samples of identical composition at each pH evaluated. (B) The effect of NaCl concentration on the OD of salmon and human fibrinogen clotted with salmon thrombin. Samples consisted of 1-u salmon thrombin, 1-mg/ml salmon or human fibrinogen, 0–500-mM NaCl, 3-mM  $\text{CaCl}_2$ , 6-mM  $\epsilon\text{ACA}$ , and 20-mM Tris, pH 7.5. Closed circles represent fibrin clots made with salmon fibrinogen, and open circles represent fibrin clots made with human fibrinogen. Measurements are based on the mean absorption value of three measurements of identical composition at each NaCl concentration evaluated.

### 2.4. Fibrin Sealant Efficiency During Wound Healing In Vivo

The potential for salmon fibrinogen to substitute for human clotting factors in biological sealant applications is supported by the mechanical properties of rat skin incisions after healing in contact with a salmon fibrin glue. Fig. 6 shows the force developed after extension of wounded rat skin treated with either human cryoprecipi-



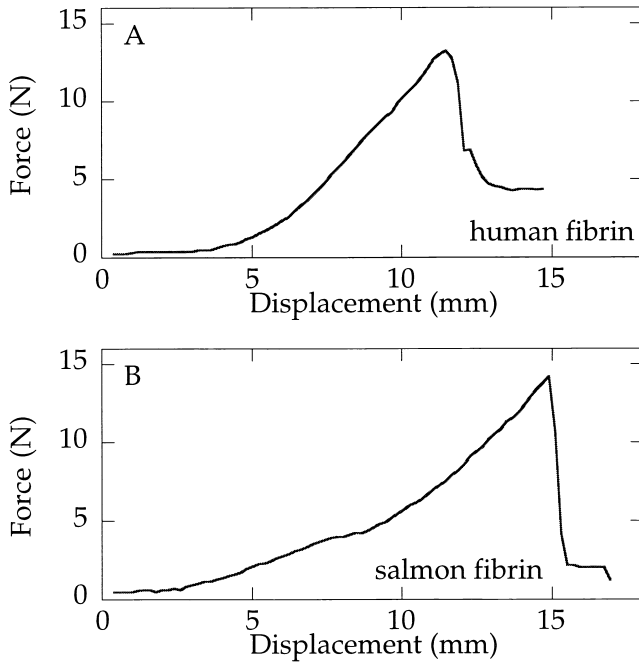


Fig. 6. The force resisting a constantly increasing elongational displacement of skin samples from rats in which lateral incisions had been healed using human cryoprecipitate (A) or salmon fibrin sealant (B).

tate (upper curve) or salmon fibrin (lower curve). The force–displacement curves corresponding qualitatively to stress–strain curves are similar within the error of measurement, and the skin sample healed in the presence of salmon fibrin survives extension to strains (approximately 40%) at least as great as those of control samples. Analysis of 15 specimens treated with salmon fibrin and 14 controls treated with human cryoprecipitate yielded values of 1.8 MPa for both samples. However, the large standard deviations of measurements within each sample group would require a difference of 1.2 MPa to allow a significant difference to be detected. When parallel samples from the same animal are compared, the standard deviations are much smaller, and again no statistically significant difference is detected.

### 2.5. Immunologic Analyses

Visual inspection of rats and mice treated with salmon fibrin glue showed no evidence of inflammation or abnormal bleeding from the wound site. None of the six rats exposed to salmon fibrin on an incision tested positive for

antibodies to fish fibrinogen using immunoelectrophoresis either 36 or 120 days after exposure. In contrast, four of five rats injected with salmon fibrinogen and Freund's adjuvant tested positive 30 days after exposure. One control rat that had not been knowingly exposed to salmon fibrinogen tested positive, possibly because of exposure to fishmeal that comprises part of the standard rat diet.

A screen of 49 normal human samples and 20 patients sera revealed two normals and four patients with potentially positive reactions to salmon fibrinogen, but four of these sera (two patients, two normals) reacted at least as strongly to purified salmon fibronectin that lacked levels of fibrinogen detectable by SDS-PAGE, suggesting that the reactivity to fibrinogen samples resulted from contamination of fibrinogen by fibronectin that is not quantitatively removed by affinity chromatography with gelatin-Sepharose [18]. One patient with antibodies of IgG type also had IgA type antibodies against both antigens. When 84 additional normal sera and 19 autoimmune sera were studied for anti-fibronectin antibodies using purified salmon fibronectin as antigen, 13 positive reactions were found.

### 3. Discussion

Fibrin sealants have been widely used in surgical procedures over the last several decades, although the growth in their use has been hampered by the unavailability until recently of a standardized commercial source. Fibrin sealants may be used as adhesives or sealants to prevent leakage, and some data support their contribution to wound healing [2,4,19] or as a controlled release system [20]. Fibrin sealants are composed primarily of fibrinogen and thrombin. Some commercial sealants available in Europe, and now one in the US, have also incorporated an anti-fibrinolytic agent to prolong the life of the polymerized clot. Fibrin sealants may be used to promote clot formation, to seal tissues, or theoretically as a drug delivery system. Hence, cardiovascular surgical uses have focused on decrease in blood loss following cardiac bypass surgery, whereas in neurosurgery, the goal is to decrease leakage of cerebrospinal fluid following

dural closure [21]. Until recently, no commercial sources of fibrin sealants were available, leading many investigators to use cryoprecipitate from either the patient or blood bank sources as the origin of the fibrinogen and bovine thrombin to catalyze clot formation. Two areas of concern raised with such materials are the dangers of infection by bacteria, viruses, or prions derived from the blood source, and the development of antibodies to the exogenous proteins that cross-react with the host protein and lead to clotting disorders. Hence, alternatives to the traditional sources of human and bovine blood products are warranted.

Clotting factors in fish blood have not yet received attention for human applications for several reasons. First, only in the past few years have the dangers, both real and perceived, from human and bovine blood products been publicized. Second, not until the recent establishment of commercial scale aquaculture have large quantities of aseptic fish blood of consistent quality been available. The blood of farmed, domesticated stocks of salmon offers an unexplored, innovative source of clotting factors free of mammalian viruses and prions.

The rapid blood clotting in most bony fishes including the Atlantic salmon (*Salmo salar*) has been well known for many years. The mechanism is a cascade of clotting factors ending in thrombin converting fibrinogen to fibrin similar to that in mammals [22]. The low ambient temperature and the relatively remote phylogeny of fishes are strong barriers to trans-species infectious agents that could be a danger to humans [23]. However, these same factors might also suggest that salmon fibrinogen may not function normally in human or mammalian applications, and the experiments reported in this study were undertaken to evaluate this possibility.

Purification and characterization of fibrinogen [12], fibronectin [18], and factor XIII [12,24] from the blood of teleost fish have been reported, but the primary structures of these proteins and their properties relative to those of human isoforms are not fully defined. The in vitro characterization of fibrinogen purified from salmon blood in the present study shows that the structure of salmon fibrin clots, their rheology, and their adhesion to biological surfaces are very similar

to that of human or bovine fibrin, as is the reactivity of salmon fibrinogen with salmon and human thrombins. Moreover, the data from Fig. 4 show that the structure of salmon clots is relatively insensitive to changes in pH and ionic strength, suggesting that salmon fibrin gels may have advantages over mammalian products in contexts where wound healing occurs at surfaces exposed to non-physiologic conditions such as the surface of dressings or sites of perfusion.

In addition to the similarity between salmon and mammalian fibrin clots formed in vitro, salmon fibrin sealants also function well in animal wound healing studies. Following the method of Lontz et al. [25] who validated the rat skin model for evaluating fibrin sealants for in vivo tests, a comparison of wounds healed in contact with salmon fibrin sealant and human cryoprecipitate showed no discernible difference between the two systems and acceptable mechanical strength of the repaired tissues in both cases.

Whereas less trans-species infection might be expected from salmon than from mammalian blood sources, the probability of immune response may be higher due to the phylogenetic difference. However, none of the preliminary assays for immune response showed any unusual antigenicity of the salmon fibrin sealant. None of the rat wound sites developed an inflammatory response, and none of rats exposed to salmon fibrin on an incision tested positive for antibodies to fish fibrinogen as long as 120 days after exposure. A search for human serum autoantibodies to salmon proteins revealed some reactivity with salmon fibronectin, but no clear specificity for fibrinogen. While prolonged exposure to salmon fibrinogen and other clotting factors is likely to produce an immune response in humans, cross-reactivity of anti-salmon fibrinogen with human fibrinogen is unlikely to be any greater than cross-reactivity to antibodies formed against clotting factors from more closely related mammalian sources. In a study of cardiothoracic surgery patients treated with bovine fibrin, all of 21 patients developed antibodies to bovine fibrinogen, but none of these antibodies cross-reacted with human fibrinogen nor did any patients develop bleeding complications even with high titers of anti-

bovine fibrinogen antibody [26]. In contrast, antibodies developed after exposure to bovine thrombin pose a significant risk for bleeding complication due to their potential to cross-react with human thrombin and factor V [27]. Thrombin and other clotting factors purified from salmon blood may pose less of a risk if the species difference makes cross-reactivity with human proteins less probable.

In summary, the blood plasma of farmed salmon is an excellent source of material for fibrin sealants that have potential for use in clinical applications. Salmon fibrinogen and factor XIII retain function at mammalian temperatures and form clots with structures and mechanical properties similar to those of human fibrin that function well in animal wound healing studies. Potential advantages of salmon plasma as a source of fibrin sealant include the relatively high fibrinogen concentration (385 mg/dl), the low ambient temperature of the fish, and the availability of large quantities of aseptic blood of uniform quality from farmed stocks. While the possibility of immune response has only been addressed in preliminary studies, the excellent material properties and significantly lower risk of trans-species infectious agents suggest that further development of cold-water fish blood as a source for fibrin sealants and other proteins may be warranted.

---

*This work has been supported in part by the NIH-NHLBI through SBIR R43 HL59032 (ESS) FIRCA grant R03 TW00787 and AR 38910 (PAJ).*

---

## References

- Gibble JW, Ness PM. Fibrin glue: the perfect operative sealant? *Transfusion* 1990;30:741–7.
- Alving BM, Weinstein MJ, Finlayson JS, Menitove JE, Fratantoni JC. Fibrin sealant: summary of a conference on characteristics and clinical uses. *Transfusion* 1995;35:783–90.
- Radosevich M, Goubran HA, Burnouf T. Fibrin sealant: scientific rationale, production methods, properties, and current clinical use. *Vox Sang* 1997;73:133–43.
- Martinowitz U, Spotnitz WD. Fibrin tissue adhesives. *Thromb Haemostasis* 1997; 78:661–6.
- Spotnitz WD, Welker RL. Clinical uses of fibrin sealant. In: Mintz PD, editor. *Transfusion therapy: clinical principles and practice*. Bethesda, MD: AABB Press.
- Doolittle RF. Fibrinogen and fibrin. *Annu Rev Biochem* 1984;53:195–229.
- Binnie CG, Lord ST. The fibrinogen sequences that interact with thrombin. *Blood* 1993;81:3186–92.
- Mosesson MW. Fibrin polymerization and its regulatory role in hemostasis. *J Lab Clin Med* 1990;116:8–16.
- Martinowitz U, Saltz R. Fibrin sealant. *Curr Opin Hematol* 1996;3:395–402.
- Will RG, Ironside JW, Ziedler M, Cousens SN, Estibeiro K, Alperovich A, Poser S, Pocchiari M, Hofman A, Smith PG. A new variant of Creutzfeldt–Jakob disease in the UK. *Lancet* 1996;347:921–5.
- Mosher DF, Blout ER. Heterogeneity of bovine fibrinogen and fibrin. *J Biol Chem* 1973;248:6896–903.
- Murtaugh PA, Halver JE, Gladner JA. Cross-linking of salmon fibrinogen and fibrin by factor XIII and transglutaminase. *Biochem Biophys Res Commun* 1973;54:849–55.
- Janmey PA, Hvidt S, Kas J, Lerche D, Maggs A, Sackmann E, Schliwa M, Stossel TP. The mechanical properties of actin gels. Elastic modulus and filament motions. *J Biol Chem* 1994;269:32503–13.
- Janmey PA. A torsion pendulum for measurement of the viscoelasticity of biopolymers and its application to actin networks. *J Biochem Biophys Methods* 1991;22:41–53.
- Janmey P, Amis E, Ferry J. Rheology of fibrin clots: VI. Stress relaxation, creep, and differential dynamic modulus of fine clots in large shearing deformations. *J Rheol* 1983;27:135–53.
- Shah JV, Janmey PA. Strain hardening of fibrin gels and plasma clots. *Rheol Acta* 1997;36:262–8.
- Kisand K, Salupere V, Uibo R. Enzyme-linked immunosorbent assays for the determination of IgG, IgA, and IgM autoantibodies to pyruvate dehydrogenase in

- primary biliary cirrhosis. *Int J Clin Lab Res* 1994;24:98–101.
18. Kurokawa T, Suzuki T, Funakoshi S. Purification of flounder (*Paralichthys olivaceus*) fibronectin and its localization during re-epithelialization at fin wound. *J Fish Biol* 1993;43:421–32.
  19. Spotnitz WD. As originally published in 1990: four years' experience with fibrin sealant in thoracic and cardiovascular surgery. Updated in 1998. *Ann Thorac Surg* 1998;65: 592–3.
  20. Cheng H, Fraidakis M, Blomback B, Lapchak P, Hoffer B, Olson L. Characterization of a fibrin glue-GDNF slow-release preparation. *Cell Transplant* 1998;7:53–61.
  21. Yoshimoto T, Sawamura Y, Houkin K, Abe H. Effectiveness of fibrin glue for preventing postoperative extradural fluid leakage. *Neurol Med-Chir* 1997;37:886–90.
  22. Doolittle R, Surgenor D. Blood coagulation in fish. *Am J Physiol* 1962;203:964–70.
  23. Wolf K. Fish viruses and fish viral diseases. Ithaca, NY: Cornell Univ. Press, 1988 (472 pp.).
  24. Monsonego A, Mizrahi T, Eitan S, Moalem G, Bardos H, Adany R, Schwartz M. Factor XIIIa as a nerve-associated transglutaminase. *FAS-EB J* 1998;12:1163–71.
  25. Lontz JF, Verderamo JM, Camac J. Assessment of restored tissue elasticity comparing fibrin sealant with suturing in prolonged in vivo animal testing. In: Alving B, Weinstein M, editors. Bethesda, MD: National Institutes of Health, 1994. p. 90 (Dec 8–9).
  26. Carroll JF, Moskowitz KA, Edwards NM, Hickey TJ, Rose EA, Budzynski AZ. Immunologic assessment of patients treated with bovine fibrin as a hemostatic agent. *Thromb Haemostasis* 1996;76:925–31.
  27. Banninger H, Hardegger T, Tobler A, Barth A, Schupbach P, Reinhart W, Lammle B, Furlan M. Fibrin glue in surgery: frequent development of inhibitors of bovine thrombin and human factor V. *Br J Haematol* 1993;85:528–32.