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Fibrin Formed in Plasma Is Composed of Fibers More Massive than those Formed from Purified Fibrinogen

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Key words

Fibrin – Plasma – pH – Ionic strength – Calcium

Summary

Reports of altered fibrin resulting from interactions with plasma proteins and cellular release products have raised the possibility that plasma fibrin may differ from purified fibrin. To investigate this possibility, the structures of thrombin-induced gels formed from platelet poor plasma and from purified fibrinogen were compared using turbidity and gel perfusion techniques. Plasma gels formed more slowly and were composed of fibers two to four times more massive than gels formed from purified fibrinogen. Increasing calcium concentration, decreasing ionic strength, decreasing thrombin concentration, or increasing fibrinogen concentration resulted in increasing fiber size. Addition of excess thrombin accelerated plasma gel formation and decreased gel fiber size, but did not totally eliminate the structural differences between the two systems. Thus, antithrombin activity, while possibly contributory, is not solely responsible for the altered gel structure. Penetration of plasma gels by fibrinolytic agents, egress to areas of injury by inflammatory cells, and gel removal by plasmin are processes at least partially dependent on gel fiber and pore size.

Introduction

While the structure of gels formed from solutions of purified fibrinogen have been the focus of intensive investigation for the past forty years, the structure of plasma gels have rarely been studied. Given the complexity of the plasma system and the relatively early development of techniques for purifying fibrinogen, the simplification of the system is understandable (1, 2). In addition, many of the techniques utilized to study protein structures such as electron microscopy and light scattering are either better suited or completely restricted to solutions of purified proteins. Recently, altered fibrin structure secondary to interactions with plasma components have been reported (3–8). When added to solutions of clotting fibrin, thrombospondin and immunoglobulins cause gel fibers to be thinner or less massive (3, 4). A similar function attributed to albumin (5) may be the result of albumin mediated calcium celation (6). Platelet factor 4, β-thromboglobulin, and leukocyte cationic protein all result in thicker, more massive fibers (7, 8). Such studies have raised the possibility that the structure of plasma-derived fibrin gels may be significantly different from gels of purified fibrin. Such differences may be important to the physiologic properties of the gel and the ability of cells (macrophages), proteins (plasminogen), and pharmologic agents (streptokinase, urokinase and tissue plasminogen activator) to penetrate the structure. In our laboratory we have utilized several physical chemical techniques to probe the structure of intact fibrin gels. With several minor modifications these same techniques can be applied to plasma gels. We report here the results of a study comparing the structures of thrombin-induced plasma and purified fibrin gels.

Materials and Methods

Human fibrinogen purified by a modified Cohn method was purchased as a lyophilized powder from KabiVitrum. It was dissolved in water and dialyzed against 0.3 M NaCl for 24 hours to remove free calcium. After dialysis, the solution was divided into 1 ml lots and stored at −90 °C. Clottability was 95% by the method of Laki (9). Fibrinogen concentration was determined spectrophotometrically from the absorbance at 280 nm using an extinction coefficient of 1.6 ml/(mg cm).

Human thrombin, greater than 90% alpha, was purchased as a lyophilized powder from Sigma Chemical Company. The material with a specific activity of 4300 NIH units/ml was dissolved in water, diluted with 0.10 M NaCl to a final concentration of 20 units/ml, divided into 1 ml lots, and frozen at −90 °C. Thrombin was free of plasmin and plasminogen. Nanopure water was used in the preparation of all solutions.

Human blood was obtained in citrated glass tubes by sterile venipuncture of normal volunteers. It was spun at low speed to remove large formed elements and then respun at 20,000 g for 20 minutes to remove platelets. Platelet poor plasma was then pooled and either frozen or maintained in an ice water bath for immediate use. The fibrinogen concentration of plasma samples was determined by the modified method of Clauss (10).

Fibrin gels were formed by rapidly mixing purified fibrinogen, or one to one dilutions of plasma, with a buffered thrombin solution (1.00 NIH/ml). Gels were formed at room temperature (25 °C) at a pH of 7.4 (0.05 M Tris). The ionic strength was routinely maintained at 0.15, except when ionic strength was the variable under study, by varying the amount of NaCl. 5 mM calcium chloride was added to all gels.

Perfusion Measurements

Gels for perfusion measurements were formed in a glass tube mounted vertically in a clamp. Both ends of the tube were equipped with a silk screen to support the gel during the measurement. The lower end was temporarily covered with parafilm to prevent leakage of solution prior to gelation. Once gelation had proceeded for at least one hour, the parafilm was removed, and the lower end of the gel-filled tube immersed in a beaker filled with perfusing solution. The upper end of the glass tube was connected to a simple perfusion apparatus the details of which have been previously reported (11). Perfusing liquid was of the same ionic strength as the medium in which the gel was formed.

Adherence of the gel to the sides of the glass tube is requisite for accurate and precise results. Good gel attachment, was assured by etching the glass tubes and by coating the tubes with a thin layer of fibrin prior to formation of the test gel. Details of these procedures have been previously reported (11).

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The permeability or Darcy constant of a gel, D, was calculated as follows:

$$D = Q\eta h/Ftp \quad (1)$$

were Q is the volume passed through in time t; η the viscosity of the liquid; h the length of the gel column; F the cross-sectional area perpendicular to the flow, and p the applied pressure. Average fiber diameter (d) was calculated from the Darcy constant as follows (12):

$$d^2 = k\varphi D \quad (2)$$

where k is a constant, and φ is the volume fraction of the polymer. A value of 10 has been empirically determined for k from measurements of permeation through irregularly packed metal rods of known diameter. This value has been confirmed by studies of wool, cotton, and cellulose fibers (13–16) and has been shown to be applicable to fibrin (11). The fiber mass-length ratios (μ) were calculated from the Darcy constant as follows (11):

$$\mu = C/(4[10\pi D]) \quad (3)$$

Where C is the concentration of fibrin in g/cm^3. The units of equation 3 are g/cm and were converted to Da/cm by multiplying by 5.988×10^{23} Da/g.

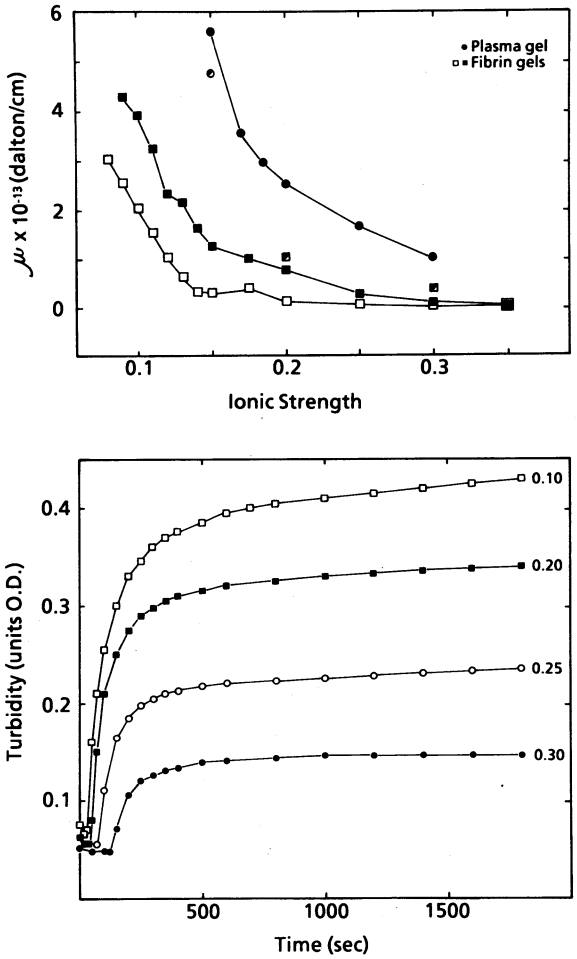

Fig. 1 Effect of ionic strength on the assembly and structure of thrombin-induced fibrin gels. Upper panel – fibrin fiber mass-length ratios of plasma (○, ●) and purified fibrin (□) gels as a function of ionic strength. The closed symbols contain 5 mM $CaCl_2$. Other clotting conditions included: pH 7.4, 1.0 NIHu thrombin/ml, and 1 mg fibrinogen/ml. All determinations were made from turbidity measurements except for the hatched (◐, ◪) symbols which represent perfusion measurements. Lower panel – kinetics of turbidity development during thrombin-induced gelation of plasma gels at the indicated ionic strengths

Turbidity Measurements

Fibrin gels for turbidity measurements were formed directly in 10 mm polystyrene cuvettes (Fisher Scientific Co.) by mixing purified fibrinogen solutions with buffered solutions of thrombin (1.0 NIHunit/ml). Plasma gels were formed in a similar fashion by adding 1.0 ml of plasma to 1.0 ml of buffered thrombin. Unless otherwise stated, gels were formed at pH 7.4 (0.05 M Tris) and ionic strength 0.15. Added $CaCl_2$ concentration was normally 5 mM.

Turbidity measurements were made at 25 °C with a Cary 2290 spectrophotometer. Kinetic measurements were made at the HeNe laser line, 632.8 nm. Fibrinogen was added to the clotting solution at time zero. Turbidity was monitored for ten minutes after which time gelation was allowed to go to completion unobserved. After 24 hours the gels were scanned from 400 to 740 nm and the mass-length ratios of the fibrin fibers were determined according to the following equation (17, 18):

$$\tau = ([88/15]\pi^3 n[dn/dc]^2 C\mu)N\lambda^3 \quad (4)$$

where n is the solution refractive index, dn/dc the refractive index increment, λ the wavelength, C the concentration of fibrinogen in g/ml, N Avogadro's number, and μ the mass-length ratio. For clear gels, μ was determined from the slope of a plot of τ versus $1/\lambda^3$ according to equation 4. As gel turbidity increases, the plot of τ versus $1/\lambda^3$ no longer extrapolates to zero. For more turbid gels where the radius of the fibers is no longer small relative to the incident wavelength, the following expression becomes appropriate:

$$(c/\tau\lambda^3) = A\mu^{-1} + B(r^2/\mu)\lambda^{-2} \quad (5)$$

where

$A = [(88/15)\pi^3 n(dn/dc)^2/N]^{-1}$

and

$B = (92\pi^2 n^2/77)A$

μ was obtained from the inverse of the intercept of a plot of $c/\tau\lambda^3$ versus $1/\lambda^2$ (19).

Results

The effects of ionic strength on plasma clotting are summarized in Figure 1. Kinetics of turbidity increase upon addition of thrombin are shown in the lower panel. As the ionic strength is decreased, the lag phase shortens, the initial slope of the turbidity rise increases, and the final gel turbidity is enhanced. The dependence of gel fiber mass-length ratios of plasma and purified fibrin gels on ionic strength is illustrated in the upper panel. For both systems the fibers become more massive as the ionic strength decreases. As reported previously, the effect of calcium has an independent but additive impact, with the fibers being consistently larger in the presence of 5 mM $CaCl_2$ at any given ionic strength. The results of several perfusion measurements on both purified fibrin and plasma gels are included to demonstrate the excellent agreement between the two techniques. Below an ionic strength of 0.15, fiber mass-length ratio is exquisitely sensitive to further decreases in ionic strength. Plasma gel fibers are 2 to 4 times larger than purified fibrin fibers under all conditions tested.

The effects of calcium on plasma gel clotting are seen in Figure 2. As the calcium concentration is increased the lag phase shortens, the initial slope of turbidity rise increases, and the final gel turbidity is enhanced (lower panel). Lag phase measurements were performed at scan rates faster than those indicated in Figure 2, but the data have been compressed for uniformity. In both systems the gel fiber size increases with increasing calcium concentration. Calcium impact is greater in the plasma system with μ increasing from 2.4 to 12.5×10^{13} as calcium is increased from 2 to 15 mM. μ in the purified system increases from 1.3 to 4.8×10^{13} over the same calcium range. Calculated fiber radii increase from 90 to 190 nm in the plasma system and from 70 to 130 nm in the purified system as calcium increased from 2 to 15 mM.

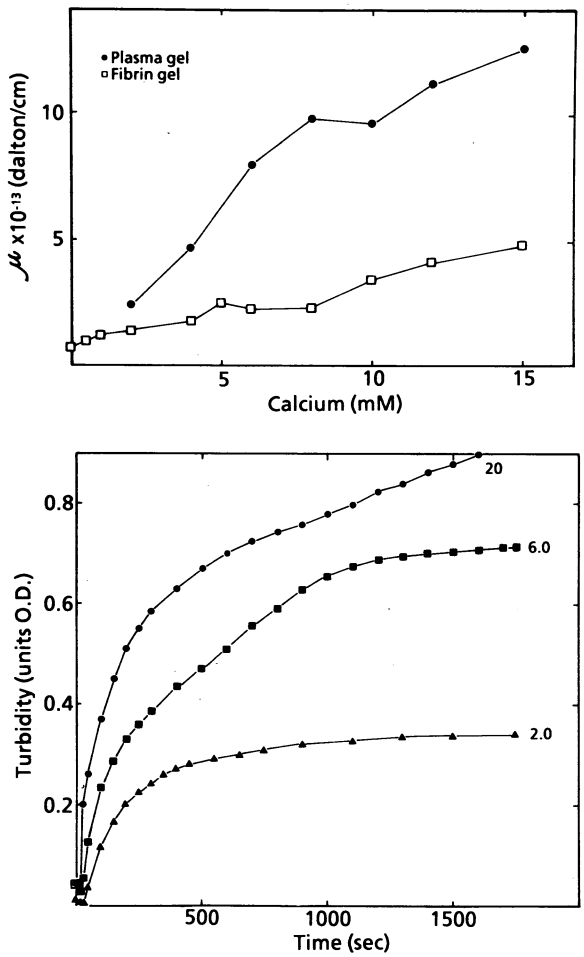

Fig. 2 Effect of calcium on the assembly and structure of thrombin-induced fibrin gels. Upper panel – fibrin fiber mass-length ratios of plasma (○) and purified fibrin (□) gels as a function of calcium. Other clotting conditions are the same as a for figure 1. All determinations were made from turbidity measurements. Lower panel – kinetics of turbidity development during thrombin-induced gelation of plasma gels at the indicated calcium concentrations (mM)

The impact of altered pH on fibrin assembly is demonstrated in Figure 3. Due to the minimal impact on plasma gel turbidity kinetics only two such plots are included in the lower panel.

Over the pH range of 7.0 to 7.4, the lag phase was minimally effected while the final gel turbidity was slightly higher at the lower pH. Below pH 7.0, fiber size and gel turbidity continued to increase (data not shown). As noted in Figures 1 and 2, plasma gel fibers were more massive under all conditions. A possible independent effect of the buffer was examined by clotting purified fibrin in both phosphate and tris buffers, lower two curves of the upper panel of Figure 3. The pH effect was virtually identical in the two systems. The data presented differ due to the absence of calcium in the phosphate system. When calcium is removed from the tris system the curves are superimposable.

As the thrombin activity is increased, fibrin fibers become thinner both in the purified and plasma systems (Fig. 4 – upper panel). The effect is more dramatic in the plasma system, the fibers being massive at low (0.125 NIHu/ml) thrombin concentrations. The kinetics of turbidity increase in plasma gels is also strongly dependent on enzyme concentration (lower panel). As the thrombin concentration is increased the lag phase is shortened, the initial slope of turbidity rise is increased, but the final gel turbidity is decreased in agreement with the decreased fiber mass-length ratios noted in the upper panel. As the thrombin concentration falls below 1.0 NIHu/ml, a biphasic component to the kinetics is noted. At about 900 seconds, the rate of turbidity rise increases. The degree of secondary increase appears to vary with the initial thrombin concentration, but the timing of its occurrence was stable over the thrombin concentrations studied. Increasing the thrombin concentration above 2 NIHu/ml did not cause plasma fibrin fibers to become as thin as purified fibrin fibers. Additional small decreases in plasma fibrin fiber size occurred as the thrombin concentration was increased to 4 NIHu/ml, but at no point did the plasma fibers become less than twice the size of purified fibrin fibers. Above 4 NIHu/ml thrombin, the plasma fibrin size actually began to increase.

The impact of sodium citrate on clotting of purified fibrin was studied by comparing gels formed in the presence and absence of sodium citrate. At all calcium concentrations tested, the fibers of gels formed in the presence of citrate (0.38 g/dl) were less massive than those formed in its absence. Measurements of free calcium concentration utilizing a calcium electrode (Orion Research Model 93-20) revealed that citrate induced differences were due to chelation of calcium by citrate. When corrected for the amount of calcium bound to citrate, gels formed in the presence of citrate were virtually identical to those formed in its absence.

Two possible sources of artifact were excluded by control experiments. First the effect of freezing on plasma samples was evaluated by comparing experimental results obtained from fresh plasma versus those obtained from freshly thawed fresh frozen plasma. The structures formed were virtually identical. The

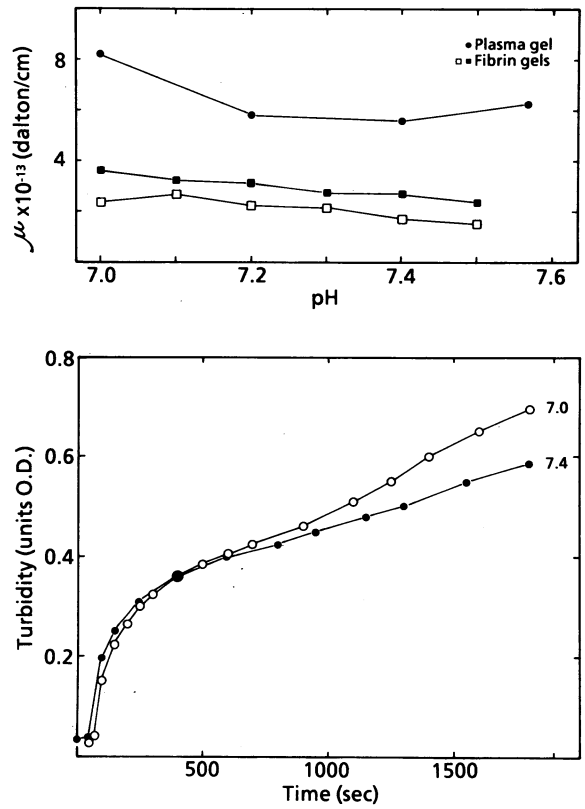

Fig. 3 Effect of pH on the assembly and structure of thrombin-induced fibrin gels. Upper panel – fibrin fiber mass-length ratios of plasma (○) and purified fibrin (□) gels as a function of pH. Closed symbols contain 5 mM $CaCl_2$. Other clotting conditions are the same as for figure 1. All determinations were made from turbidity measurements. Lower panel – kinetics of turbidity development during thrombin-induced gelation of plasma gels at the indicated pH

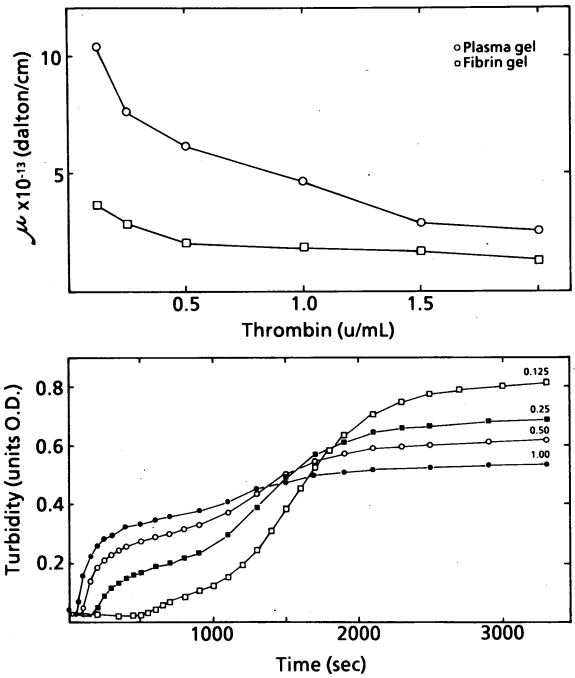

Fig. 4 Effect of thrombin concentration on the assembly and structure of thrombin-induced fibrin gels. Upper panel – fibrin fiber mass-length ratios of plasma (○) and purified fibrin (□) gels as a function of thrombin concentration. All gels contain 5 mM $CaCl_2$ and other conditions are as for figure 1. All determinations were made from turbidity measurements. Lower panel – kinetics of turbidity development during thrombin-induced gelation of plasma gels. Thrombin concentrations (NIHu/ml) are indicated at the right

frozen samples could not, however, be refrozen without altering the subsequent gel structure. Second the possibility of small differences in ionic environment of plasma and fibrinogen solutions, caused by our mixing system, was evaluated by comparing the above results with those obtained from samples where the ionic environment was set by dialysis. The structures of gels formed from dialyzed samples were comparable to those seen with mixed samples, and the differences between purified and plasma fibrin were still apparent.

Discussion

In a purified system, numerous variables alter fibrin fiber size. Positively charged molecules including calcium, platelet factor 4, leukocyte cationic protein, protamine, and synthetic polymers cause increased fiber diameter (7, 8, 20, 21). Large carbohydrate polymers such as dextran and hydroxyethyl starch also cause fiber enlargement (17, 18, 22). Conversely, thrombospondin and high levels of immunoglobulins cause decreased fiber diameter (3, 4). The addition of platelet extracts to purified fibrin reportedly leads to thinner fiber production (23). Factor XIIIa, known to induce covalent bonds into fibrin, and fibronectin, known to be incorporated into fibrin, have surprisingly minimal effect on fiber assembly or size (24). Antithrombin activity may be partially responsible for increased plasma fibrin fiber enlargement. When excess thrombin is added to plasma, the fibrin fibers are thinner. The combined effects of these, and possibly other, parameters is thick plasma fibrin fiber production.

The data from this study are in agreement with results of a recent investigation which utilized magnetic birefringence to study fibrin assembly (25). Both studies revealed that: 1) fibers formed from citrated plasma were much larger than fibers in purified fibrin, 2) fibronectin has little or no effect on fiber assembly, 3) Factor XIIIa does not effect clot assembly, and 4) assembly induced by reptilase is similar to that seen with thrombin. Plasma fibrin polymerization kinetics as seen with magnetic birefringence demonstrate the biphasic effects seen with turbidity. The impact of thrombin concentration on assembly kinetics is similar by both techniques. A major difference between the studies is the demonstration by magnetic birefringence that albumin causes a thickening of fibrin fibers. If this is truly the case, albumin may be partially responsible for the thicker fibers seen in plasma. We are unable to confirm this result in our system (6). Indeed, in systems utilizing higher thrombin concentrations, albumin seems to have no impact (6, 26), while in low thrombin systems albumin appears to speed polymerization (25, 27). Some investigators even report thinner fibrin fibers in the presence of albumin (5). At the very least, the impact of albumin remains controversial.

Altered fibrinopeptide release in plasma is not directly considered in this study, but its possible impact on fibrin structure is minimized by previous investigations. Pertinent findings include the following: reptilase, which releases only fibrinopeptide A, clots fibrinogen (28, 29, 30); the majority of thrombin-induced fibrinopeptide B release occurs after network formation (31); fibrin fibers formed by thrombin and reptilase are virtually identical (20); and fibrin formed from a varient fibrinogen with delayed fibrinopeptide A release has a normal structure (32). Thus, clot structure is similar regardless of the sequence of fibrinopeptide removal and is predominately a function of the ionic environment in which it forms.

Finally, it is possible that fibrin formed in plasma might be influenced by the presence of other clotting factors. We recently investigated this possibility, and found that plasma fibrin formed by activation of the intrinsic clotting system is identical to fibrin formed by the direct addition of thrombin (33). In both situations, plasma fibrin is composed of fibers larger than those formed from purified fibrinogen.

The implications of thick fiber structure should be considered from several perspectives: its impact on fibrin's ability to serve as a substrate, its impact on gel structural properties, and its impact on the ability of cells and subcellular materials to enter the gel. It is known, for example, that thicker fibers induced by dextran leads to more rapid clot lysis by plasminogen (34–36). Whether this is the result of altered plasmin binding or enhanced plasminogen activation is not known. It is also known that gels composed of thicker fibers are more rigid, and less friable (2). Such properties may be of importance given fibrin's hemostatic role.

As fiber size increases, pore size increases. If two gels of equal fibrin concentration are formed under differing conditions, such that one gel is composed primarily of thick fibers and the other is composed of thin fibers; the gel containing thick fibers will have larger pores. As gel fibers increase in diameter, the fibrin contained in a given volume is concentrated into a relatively small portion of the volume while the remainder of the volume becomes relatively devoid of fibrin. Recent studies involving whole blood clots have confirmed this hypothesis (37). When fibrin fibers are large, the pore sizes are large, and intact erythrocytes can be washed from the network. When fibrin fibers are thin, pore sizes are small, and erythrocytes are trapped within the network. Thick fibers and larger pores may be requirements of a gel structure allowing egress of materials critical for clot removal and wound healing.

Acknowledgements

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