Supplementary Information

Fibrin Clots Are Equilibrium Polymers That Can Be Remodeled Without Proteolytic Digestion

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Effect of crosslinking on FRAP of fibrin

To examine the effect of crosslinking on FRAP of fibrin, clots were made from purified fibrinogen with added thrombin as described above in the presence of 0.34 mg/ml or 0.022 mg/ml Factor XIII (Fig S1C). In these experiments, no recovery of fluorescence was observed (Fig. S1A). Similarly, platelet-poor plasma clots without the Factor XIII inhibitor were also studied. Again, no recovery of fluorescence was observed (Fig. S1B).

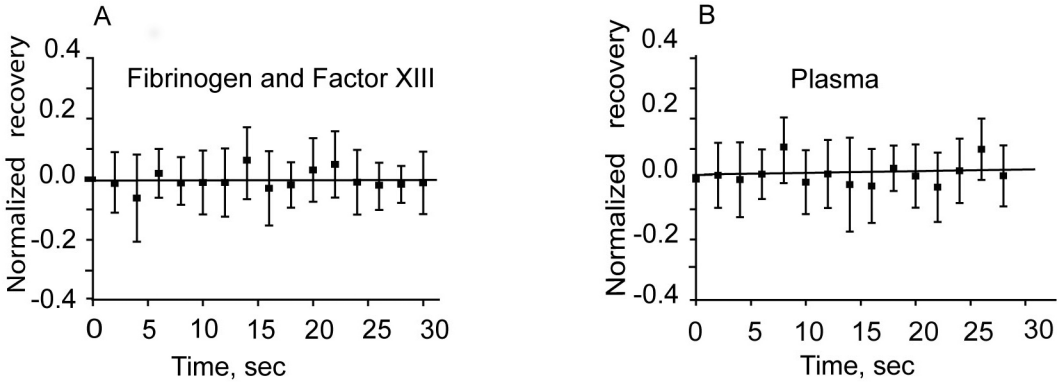

Fig S1: (A) No recovery of fluorescence is observed in purified fibrin clots in the presence of Factor XIII. (B) In platelet poor plasma clots, no recovery of fluorescence is observed in the absence of Factor XIII inhibitor. (C) SDS PAGE showing crosslinking of fibrin by physiological levels of Factor XIII (22 μg/ml). From left to right, the lanes contain fibrin clots at 0, 15, 30, 45, 60 min, and molecular weight standards. At 0 min, the bands represent α, β and γ chains, and then γ dimer and α multimer bands appear as a function of time.

Theoretical basis of FRAP experiments

The FRAP experiments investigate a binding reaction of the form:

$$F + S \leftrightarrow C, \qquad (1)$$

where F represents the free fibrin monomers, S represents the vacant binding sites on the immobile fibers, and C (which is FS) represents the bound complex. k_{on} and k_{off} are the on and off rates for the forward and backward reactions respectively. We assume that the reaction above has reached equilibrium before the photo-bleach is performed in the FRAP experiment[1]. The assumption of equilibrium implies that the concentrations of F, S and C have reached their constant equilibrium values F_{eq}, S_{eq} and C_{eq} by the time FRAP is performed. We also assumed that no movement of fibrin molecules occurred during photobleaching and minimum photobleaching occurs during the recovery phase. The photo-bleach changes the number of visible F and C in a region but it does not change the concentration of binding sites which remains fixed at S_{eq}. Lastly, if we assume that diffusion is very fast compared to the time scale over which binding events occur or to the timescale of the FRAP experiment, then $[F] = F_{eq}$ very quickly after the photo-bleach. To show that diffusion is fast we performed FRAP on the background solution away from the fibrin fibers in the clot. In the background solution there is a very small number of fibrin oligomers that fail to polymerize, and our goal was to measure how

fast these oligomers diffuse. We bleached a small square region 5 μm X 5 μm at several locations in the background and measured the recovery as a function of time. We found that more than 80% of the recovery occurred within a fraction of a second after the photobleach. This suggests that the fluorescent fibrin oligomers in solution diffuse into the bleached region very rapidly in comparison to the FRAP recovery times we see in fibrin fibers in the clot. In order to be more quantitative we solved the diffusion equation in two dimensions to estimate the size of the oligomers that will have a diffusion constant to fit the recovery data. The initial condition for the diffusion equation was that the concentration of fluorescent oligomers was uniform over a big square of side L, while the concentration was zero in the bleached area (in the center of the big square) of side a. The diffusion equation was solved using double Fourier series. The solution is not presented here because it is standard and can be easily found in textbooks [2,3]. We give the expression for the fluorescence recovery curve $recd(t)$ as a function of time in eqn. (2).

$$recd(t) = 1 - \sum_{n=1}^{\infty}\sum_{m=1}^{\infty} \frac{4\sin^2(\frac{n\pi a}{L})\sin^2(\frac{m\pi a}{L})}{\frac{a^2}{L^2}(1-\frac{a^2}{L^2})n^2m^2\pi^4} e^{\frac{-D(n^2+m^2)\pi^2 t}{L^2}} \qquad (2)$$

Here $D = k_B T/6\pi\eta r$ is the diffusion constant of the oligomers which are assumed to be spheres of radius r, k_B is the Boltzmann constant, T is the absolute temperature (300K) and η is the viscosity of water at room temperature. In order to obtain the black curve in Fig. S2 we have used $r = 20\,\text{nm}$ and $a/L = 400$. Using $r = 20\,\text{nm}$ gives $D = 10.867\mu m^2/s$ which is about the same as that for a pentamer. The fact that the curve matches the data well suggests that the free fibrin oligomers in the background solution are only a few tens of nanometers in size and diffuse very fast.

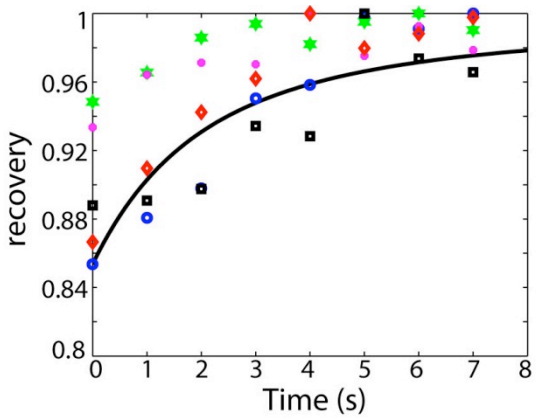

Fig. S2: We performed FRAP on the clot background at various locations away from the fibrin fibers. The recovery data from each location is shown in a different color. About 80% recovery happens in a fraction of a second because the monomers/oligomers in solution diffuse very quickly into the bleach spot which is 5 microns X 5 microns. The black line above is obtained by solving the diffusion equation in two dimensions assuming that the oligomers are spheres of radius 20 nm in water.

In such a scenario the rate at which the number of bound complexes evolves is given by,

$$\frac{d[C]}{dt} = k_{on} S_{eq} F_{eq} - k_{off}[C], \tag{3}$$

This is a first order ordinary differential equation and its solution is

$$[C](t) = C_{eq}(1 - \exp(-k_{off} t)), \tag{4}$$

where $C_{eq} = k_{on} S_{eq} F_{eq} / k_{off}$ is the equilibrium concentration of C, and we have assumed the initial condition that at $t = 0, [C](0) = 0$. Since, there is a very small concentration of fibrin oligomers in the background solution, FRAP recovery in the bleached fibers can also occur if the off-rate k_{off} is zero, but since clottability is 97%, it is not enough to account for the magnitude of recovery. Furthermore, in that case the solution of eqn. (3) will be linear in t and not exponential. In our experiments we observe an exponential recovery curve. Furthermore, since diffusion is very fast the free oligomers will get attached to the fibers quickly, but recovery will cease early because the concentration of free oligomers in solution is very small. Again, this is not observed in our experiments; recovery continues for about 25 seconds. For these reasons, we

believe that the off-rate is not zero, or the fibrin polymerization reaction is not irreversible. So, the fluorescence intensity $rec(t)$ after photo-bleaching in the FRAP experiment is given as a function of time t by the following:

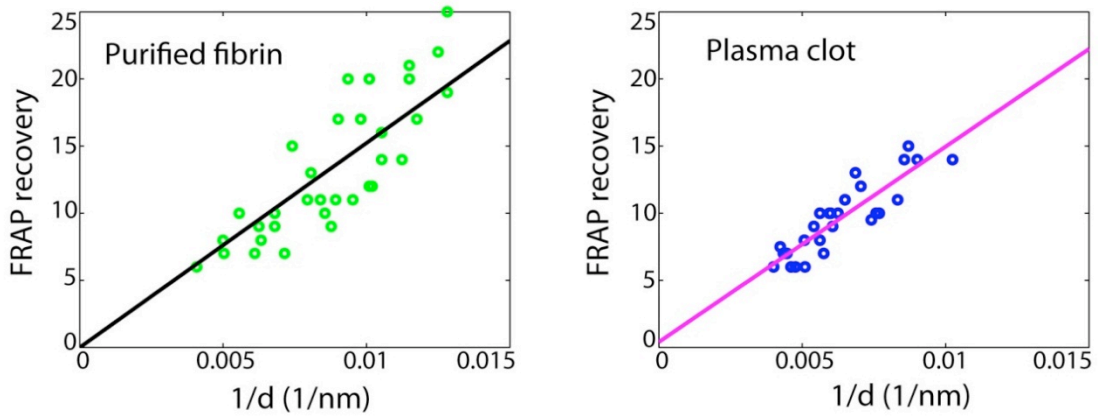

Fig. S3: The plots above show the FRAP recovery as a function of the surface to volume ratio, 1/d, of cylindrical fibers. Linear fits to the data are also shown. The lines pass through the origin for purified fibrin as well as plasma clots. This suggests that the turnover of fibrin monomers occurs mostly on the surface of the fibers.

$$rec(t) = q[F_{eq} + C(t)] = B[k_{off} + k_{on} S_{eq}(1 - \exp(-k_{off} t)], \quad (5)$$

where q is a constant that relates fluorescence intensity to the concentration of the fluorescent species, and $B = qF_{eq} / k_{off}$ is another constant. We fit the FRAP recovery curves using this formula and obtain the values of the off-rate k_{off} and qC_{eq}. In the figures we plot qC_{eq} (which is a fluorescence intensity) as a function of the fiber diameter and GPRP concentration; since q is assumed constant qC_{eq} represents the concentration of bound complex consisting of fibrin monomers bound to sites on a fibrin fiber.

It is useful to determine the rate of fluorescence recovery for short times by expanding the exponential. We see that for short times

$$rec(t) = q[F_{eq} + C_{eq} k_{off} t] = qF_{eq}[1 + k_{on} S_{eq} t], \quad (6)$$

so that the initial rate of recovery is proportional to $k_{on}F_{eq}S_{eq}$.

Fitting the recovery data

The FRAP recovery data was fit with the expressions above using the least-squares method. The fitting parameters were qF_{eq}, qC_{eq} and k_{off}. The full exponential form of the recovery curve eqn. (3) was used for fitting the data from the experiments with and without perfused GPRP. For some experiments we used the linear form eqn. (5) suitable for short times and got the initial rate of fluorescence recovery by fitting a straight line to the recovery data as a function of time.

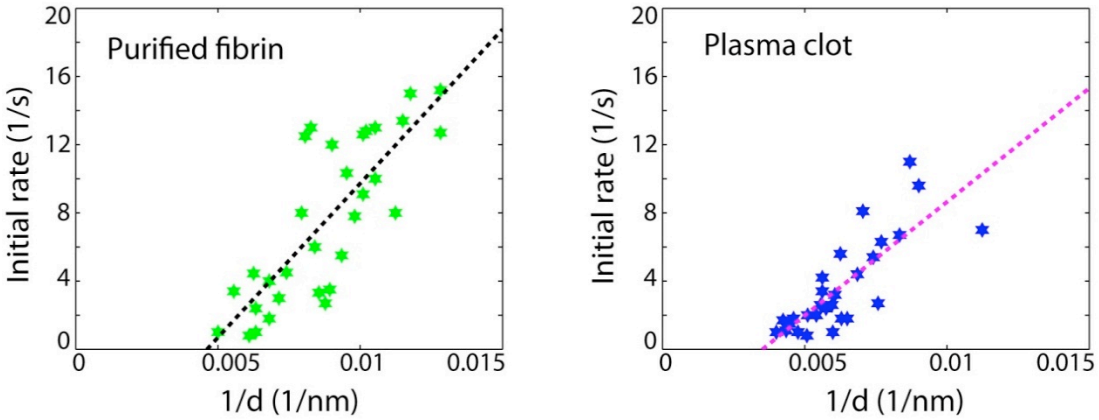

Fig. S4: The plots above show the initial rate of fluorescence recovery as a function of the surface to volume ratio, 1/d, of cylindrical fibers. Linear fits to the data are also shown. This suggests, again, that the turnover of fibrin monomers occurs mostly on the surface of the fibers.

Results from fitting the recovery data

The percentage of fluorescence recovery and the initial rate of fluorescence recovery in clots seem to depend linearly on the surface area to volume ratio of the fibers (see Fig. S3 and Fig. S4). This suggests that the binding reaction described by eqn. (1) occurs predominantly on the surface of the fibers. Here we give a quantitative explanation for why we think so. Photobleaching leads to a loss of fluorescence over the entire volume of a fiber of length l and diameter d. The number of fibrin monomers with bleached fluorescence is therefore given by $C_1\pi d^2 l$ where C_1 is a constant. Fluorescence recovery can occur due to monomers or oligomers

attaching to the surface of the fiber as well as the interior of the fiber. So, we assume that the number of monomers with recovered fluorescence is $C_2\pi dl + C_3\pi d^2 l$ where C_2 and C_3 are two other constants. We would expect C_2 to be large if most of the fluorescence recovery occurred on the surface of the fiber where as we would expect C_3 to be large if most of the recovery occurred in the interior. The fraction of fluorescence recovery is given by:

$$rec(t \to \infty) = \frac{C_2\pi dl + C_3\pi d^2 l}{C_1\pi d^2 l} = \frac{C_3}{C_1} + \frac{C_2}{C_1 d}, \qquad (7)$$

which is linear in the surface to volume ratio $1/d$ of the fiber. Note that the slope of the line gives the propensity of recovery occurring on the surface of a fiber and the intercept gives the propensity of recovery in the interior of the fiber. From our fits (see Fig. S3) we found that the straight line passed very near the origin, which made us conclude that most of the fluorescence recovery occurred on the surface the fiber. Similar reasoning can be applied to the initial rate of fluorescence recovery. From eqn. (6) we had concluded that the initial rate of recovery is proportional to $k_{on}F_{eq}S_{eq}$. Note that k_{on} and F_{eq} are constants independent of the fiber diameter. If the reaction described by eqn. (1) occurs predominantly on the surface of the fibers then the concentration S_{eq} (which is number of immobile binding sites per unit volume in a cylindrical fiber) will again be proportional to $C_4 + C_5/d$. Our linear fits (see Fig. S4) to the rate of recovery as a function of $1/d$ suggest that the turnover occurs on the surface of the fibrin fibers.

In the GPRP perfusion experiments, we found that the off-rate k_{off} seems not to be affected by the GPRP concentration. On the other hand the equilibrium concentration of the bound complex C_{eq} decreases with GPRP concentration for all fiber diameters. If we assume that the rate k_{on} remains unaffected by the GPRP concentration then our result suggests that the concentration

of immobile binding sites S_{eq} decreases as the GPRP concentration increases. This is consistent with the role of GPRP as a competitive inhibitor of fibrin fiber formation.

Additional References

1. Sprague, B. L., Pego, R. L., Stavreva, D. A. & McNally, J. G. Analysis of binding reactions by fluorescence recovery after photobleaching. *Biophys J* **86**, 3473-95 (2004).
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