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Fluorogenic Fibrinogen and Fibrin Facilitate Macromolecular Assembly and Dynamic Assay of Picomolar Levels of Plasminogen Activators under Well Mixed Conditions

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Summary

Fibrinogen labeled with fluorescein isothiocyanate (FITC) was tested for its ability to serve as a template for macromolecular assembly as well as to provide a fluorogenic signal to allow continuous monitoring of plasminogen activation and fibrinolysis. As dilute solutions of FITC-fibrinogen or FITC-fibrin fiber suspension were degraded during lysis, release of fluorescent fragments abolished proximity-based quenching and resulted in a 2.0- or 3.6-fold increase in fluorescence intensity, respectively. Addition of plasmin at a final concentration of 10 pM to FITC-fibrinogen (10 nM) produced a detectable level of fluorescence dequenching. The assay had sufficient sensitivity to detect plasmin activity in the presence of excess antiplasmin activity, indicating the dissociation of a reversible antiplasmin-plasmin complex. The detection limit of the reaction assay was 20 pM and 200 pM of recombinant tPA and urokinase, using 10 nM FITC-fibrin and 10 nM and 5 nM plasminogen, respectively. The 10-fold greater sensitivity of the assay for tPA was likely due to the molecular assembly of tPA and plasminogen on the FITC-fibrin. Addition of thrombin (1 U/ml) and plasmin (0.1 nM) to 10 nM FITC-fibrinogen produced fluorescence quenching at first due to fibrinogen polymerization followed by dequenching due to fibrinolysis. Addition of 10 mM ϵ-aminocaproic acid to mixtures of thrombin and plasmin allowed the quenching assay of thrombin activity in the presence of active plasmin. FITC-fibrinogen could be copolymerized with recalcified platelet poor plasma (isolated from citrated whole blood) to yield fibrin that was fluorogenic. Dequenching was observed when plasmin was used to degrade the fibrin formed from the platelet poor plasma. Given the large signal generated upon degradation of the fluorogenic fibrin(ogen), at least 10^5 determinations can be run from 100 mg of FITC-labeled fibrinogen using a standard fluorimeter and 0.1 to 3.0 ml reaction volumes. The versatility of the fluorogenic fibrinogen substrate allowed the configuration of assays to detect and measure the activity of thrombin, plasmin, tPA, uPA, and α_2-antiplasmin. The ability to assemble blood proteins on a fluorogenic fibrinogen or fibrin template provides unique opportunities for the dynamic study of binding and enzymatic events on the fibrin surface under well mixed conditions.

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Abbreviations: Fluorescein isothiocyanate (FITC), tissue plasminogen activator (tPA), urokinase (uPA), plasminogen activator inhibitor type 1 (PAI-1).

Introduction

The quantitation of fibrinolytic reactions is complicated by the important contributions of protein assembly on the fibrin surface as well as the difficulty in monitoring the dynamics of proteolytic events. Fluorogenic peptide substrates (1, 2) for tPA or plasmin provide greater sensitivity over chromogenic substrates, yet small peptide substrates fail to recreate the binding events that exist in the body due to the two-phase nature (fluid and fibrin) of the clot. The desirability of creating a fibrinogen or fibrin substrate that allows protein binding while providing a large signal for dynamic monitoring lysis is several fold. Fibrin gel assays have been used historically, but require daily preparation (with subsequent variations in polymerization) and may contain significant diffusion limitations which cause a coupling of lysis rates with protein penetration rates (3, 4). Analysis of molecular films of fibrinogen or fibrin, which lack diffusion limitations, is complicated by the conformational changes of these proteins upon immobilization (5). Also, the monitoring of gel or clot solubilization using the ^{125}I-release assay requires discrete sampling. Turbidity assays of plasminogen activator activity conducted on a spectrophotometer often require micromolar levels of fibrin and enzymes as well as several hours of reaction. More advanced techniques to monitor proteolysis based on light scattering (6) or fluorescence polarization anisotropy (7) are not common in the clinical setting, and often can not be used for rapid reactions.

Fluorescently labeled fibrinogen provides unique characteristics as a fluorogenic substrate since it allows molecular assembly. The multiple fluorescent labels on the dimeric fibrinogen experience proximity-based quenching. Polymerization causes additional fluorescence quenching, while plasmin mediated proteolysis releases fluorescent fragments that are dequenched and subsequently have much greater fluorescence emission (8). Our previous characterization of FITC-fibrinogen and FITC-fibrin as fluorogenic substrates to monitor plasmin activity (8) showed that the majority of fluorescence dequenching during fibrinogenolysis corresponded to the generation of fragment X and the release of carboxy terminus of α chains (αC). Recent studies based on electron microscopy analysis of fibrin by Veklich et al. (9) have demonstrated that the carboxy terminus domain of the α chains (αC) of fibrinogen are in close proximity to each other and to the E domain. The αC domains of monomers closely interact with each other during polymerization and are released during lysis. The substantial fluorescence dequenching during fragment X generation (8) and substantial quenching during polymerization are consistent with structural changes documented by statistical analysis of data from electron microscopy (9).

In the present work, we describe the use of fluorescently labeled fibrinogen and fibrin fiber suspension as substrates that allow the assembly of the fibrinolytic system on fibrin fibers while producing a large signal that is dependent on the rate of fibrinolysis, but not dependent on the sluggishness of diffusional mass transfer to move proteins into fibrin gels or plasma clots. Fibrin fiber suspensions have been used previously for the assay of plasmin and plasminogen binding without diffusion artifacts (8, 10, 11). Assays can be run using nanomolar levels of fibrin(ogen) substrate and picomolar or nanomolar levels of plasminogen activators. Under routine conditions, the assay can detect 10 pM of plasmin activity, 20 pM of tPA activity, and 200 pM of uPA activity in less than 1 h using convenient reaction volumes typical of cuvettes or 96-well plates. Additionally, the assay may be run in a reverse mode for use as a PAI-1 or α_2-antiplasmin activity assay, for example. We have found reliable detection of picomolar and nanomolar levels of blood enzymes using fluorogenic fibrinogen or fibrin that allow macromolecular assembly of the fibrin(ogen)olytic system without diffusion limitations.

Material and Methods

Reagents

Lyophilized human fibrinogen (Grade L, Kabi AB) was dissolved in 0.05 M tris(hydroxymethyl)-aminomethane (Tris-HCl) pH 7.4 and dialyzed at 4° C against 0.05 M Tris-HCl containing 0.1 or 0.3 M NaCl for 18 h with 1 l changes every 6 h. The solution was centrifuged at 2,000 × g for 20 min (4° C) and the supernatant was frozen in small aliquots at –75° C. The concentration of fibrinogen was measured spectrophotometrically at 280 nm, with $E^{1\%}_{1cm}$= 15.1 and M_r = 340,000. Purified human thrombin (Sigma) was obtained as a lyophilized powder (specific activity: 3,000 NIH U/mg). The thrombin was dissolved in high purity water at 200 NIH U/ml, aliquoted, and frozen at –75° C. Purified human glu- and lys-plasminogen (American Diagnostica Inc.) were reconstituted, centrifuged at 2,000 × g for 20 min (4° C), and stored at –75° C. Protein concentrations of glu- and lys-plasminogen solutions were determined with $E^{1\%}_{1cm}$ = 16.9 and 18.0, respectively. Human plasmin (American Diagnostica Inc.) was obtained as a lyophilized powder that had greater than 95% active sites determined using active-site titration based on a M_r = 80,000. The plasmin was dissolved in high purity water, aliquoted, and frozen at –75° C. Human α_2-antiplasmin (Calbiochem) with purity greater than 95% by SDS-PAGE was reconstituted, centrifuged at 2,000 × g for 20 min (4° C) and stored at –75° C. Recombinant human tPA was obtained as a gift from Dr. W. Bennett (Genentech, Inc., So. San Francisco, CA). Urokinase was obtained as a gift from Dr. A. Sasahara (Abbott Laboratories, Abbott Pk., IL). Recombinant human tPA and urokinase were weighed and dissolved in high purity water to give the final concentrations of stock solutions that were aliquoted and diluted at time of use. Gly-Pro-Arg-Pro peptide and ϵ-aminocaproic acid were obtained from Calbiochem. Fluorescein isothiocyanate, isomer I (FITC) was obtained from Molecular Probes (Eugene, OR). Fluorescence labeling of fibrinogen was conducted as previously described (8). Briefly, fibrinogen (10 mg/ml) was incubated with FITC (1 mg/ml) with continuous stirring for 1 h at 22° C in a labeling buffer of 0.1 M sodium bicarbonate (pH 9.0). The reaction was stopped with hydroxylamine (0.15 M final concentration) after which the FITC-fibrinogen was dialyzed extensively in the dark at 4° C and stored at –75° C. Nonreduced FITC-fibrinogen had an apparent size of 320 to 340 kD and intact α-chains as indicated by SDS-PAGE. The FITC-fibrinogen was stable for at least 15 months.

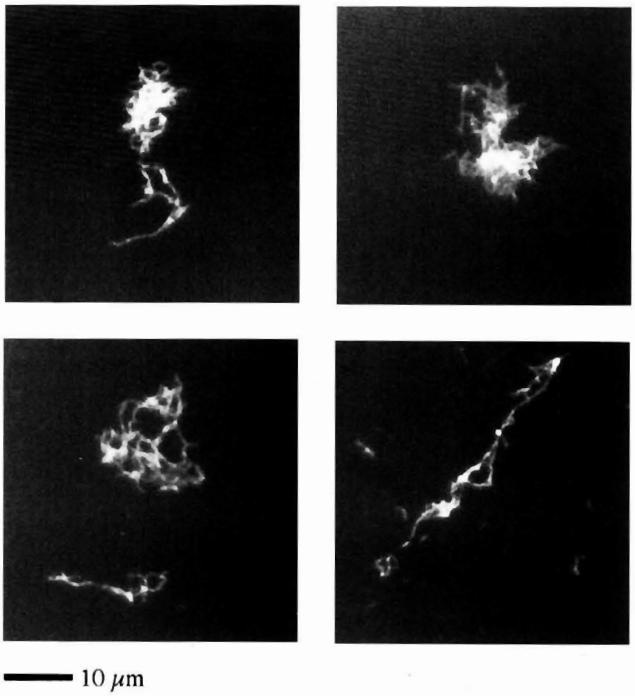


Fig. 1 Epifluorescence visualization of fibrin fibers from polymerization of dilute fibrinogen solution. Fibrin was formed by addition of thrombin (final concentration 1 U/ml) to 80 nM FITC-fibrinogen with mixing for 5 s. Polymerization of fibrinogen proceeded without stirring and was monitored via change in fluorescence for over an hour until a steady state level of fluorescence quenching was achieved. The reaction buffer was 0.1 M NaCl, 5 mM $CaCl_2$, and 50 mM Tris-HCl (pH 7.4). Small aliquots of this reaction mixture were visualized between coverslips using fluorescence microscopy

SDS-PAGE and Fluorescence Scanning

Samples removed from reactions were prepared for SDS-PAGE with prompt heating at 9° C for 5 min in SDS running buffer [TE buffer (pH 8.0), 2 % (v/v) SDS, 8 M urea, ± 1% (v/v) β-mercaptoethanol]. The samples were run on 0.5 mm thick, 4 to 15% polyacrylamide gels (Pharmacia PhastGel) and scanned using an epifluorescence microscope with a 10X objective (Leitz Aristoplan). The fluorescence emission was directed at an intensified CCD camera (Dage MTI), recorded digitally and evaluated using NIH Image 1.49 software. Following fluorescence scanning, the gels were silver stained.

Reaction Conditions and Fluorimetry

The buffer for polymerization and lysis experiments contained 0.1 M NaCl, 0.05 M Tris-HCl (pH 7.4), and 5 mM $CaCl_2$. Cuvettes were preincubated with 10 μM BSA for 30 min to minimize adsorption. FITC-fibrinogen had stable fluorescence emission with little variation for several hours. To produce suspensions of fibrin fibers, thrombin (final concentration 1 U/ml) was mixed (for 5 s) into dilute fibrinogen (80 nM) solutions at 0.1 M NaCl after which the polymerization was monitored until fluorescence quenching was complete and stable for 1 h. The daily-made fiber suspensions yielded a highly repeatable extent of quenching and were stable for several hours as indicated by the stability of the fluorescence signal. Small volumes of the fiber suspension were then pipetted into 2.4 ml of the reaction buffer and monitored for 200 to 400 s to establish the fluorescence baseline before addition of plasmin or other reagents. Fluorescence emission intensity was measured in a Perkin-Elmer LS-50 luminescence spectrometer every one or two seconds. All concentrations refer to final concentration in the reaction mixture unless otherwise noted.

Results

Thrombin-induced polymerization of a dilute solution of fibrinogen (80 nM) resulted in the generation of a suspension of fibrin fibers and loose aggregates of 5 to 10 μm in size (Fig. 1). Aliquots of this reaction mixture could be further diluted and resuspended to a final concentration of 1 to 10 nM of fibrin fiber suspensions. These small aggregates

remained freely suspended during the time course of the assay (over several hours) as indicated by the stability of the fluorescence baseline. This suspension of fibrin fibers allowed assay of fibrin degradation without the complication of diffusion limitations typically found with fibrin gels where enzyme penetration times contribute to the overall reaction phenomena.

During the polymerization of fibrinogen by thrombin, we observed a substantial quenching due to monomer-monomer interactions in the protofibril and fiber. To demonstrate the requirement for interactions between monomers, we used Gly-Pro-Arg-Pro (GPRP) to prevent assembly of fibrin monomers (12). GPRP caused a dose-dependent reduction in the rate of quenching and the final extent of quenching, with over 80% of the final extent of quenching prevented by 100 μM GPRP (Fig. 2A). To further characterize the nature of monomer-monomer interactions in the fibrin fiber, we carried out polymerization of 35 nM FITC-fibrinogen in the presence of increasing concentrations of unlabeled fibrinogen (up to 175 nM). Addition of thrombin at a final concentration of 1 U/ml caused rapid polymerization which was complete by 1 h as indicated by the final extent of quenching at all ratios of unlabeled fibrinogen/FITC-fibrinogen from 0 to 5. In the absence of unlabeled fibrinogen, the final fiber suspension had a quenched emission that was 45 and 48% of the emission prior to polymerization of fibrinogen at 0.3 or 0.1 M NaCl, respectively (Fig. 2B). The final quenched fluorescence emission was only 10% less than the original fluorescence intensity prior to thrombin addition when the molar ratio of unlabeled to labeled fibrinogen was 5.0. This indicated that the probability of interaction between fluorescent monomers was substantially reduced under these conditions. The large attenuation in quenching seen with the addition of one unlabeled fibrinogen molecule for each labeled molecule (Fig. 2B) indicated that a given monomer interacts strongly with its neighboring monomer in the protofibril, along with secondary interactions with only a few other monomers in neighboring protofibrils in the fiber. The slightly higher degree of quenching observed with fibers polymerized under low ionic strength (0.1 M NaCl, coarse) as compared to those formed under high ionic strength (0.3 M NaCl, fine) was consistent with measurements of fiber density of 210 mg-fibrin/ml-fiber for fine fibers (13) and 280 mg-fibrin/ml-fiber for coarse fibers (13, 14). However, the fibers created during polymerization of dilute solutions of 80 nM fibrinogen were likely thinner than those formed with physiologic concentrations of fibrinogen in plasma (8.8 μM). Thus, fiber density differences and the associated extent of quenching at different ionic strengths would be expected to be small as they indeed were (Fig. 2B). The FITC-fibrin substrate provided an easily measured and stable baseline signal at concentrations of 1 to 10 nM. The fluorescence signal was stable for several hours and typically had noise at a level of ± 0.25 fluorescence units. At FITC-fibrin(ogen) substrate concentrations of 1 to 10 nM and typical reaction volumes of 1 to 2 ml in cuvettes or 200 μl for 96-well plates, over 10^5 assay determinations can be conducted with 100 mg of FITC-fibrinogen.

Addition of plasmin to FITC-fibrinogen or FITC-fibrin resulted in a rapid and dose-dependent elevation in fluorescence emission that finally increased over 2.0- and 3.6-fold over the initial baseline signal, respectively (Figs. 3A and 3B). Since the FITC-fibrin was initally in a quenched state beyond that of FITC-fibrinogen as seen in Fig. 2, the fluorescence increase after lysis was greater for fibrin than for fibrinogen. We have previously demonstrated using SDS-PAGE (8) that the maximum rate of dequenching correlated with the generation of fragment X. No lag phase occurred upon addition of plasmin to either fibrinogen or fibrin. In fact, the most rapid lysis was observed immediately after addition of plasmin to fibrin. This suggests that plasmin

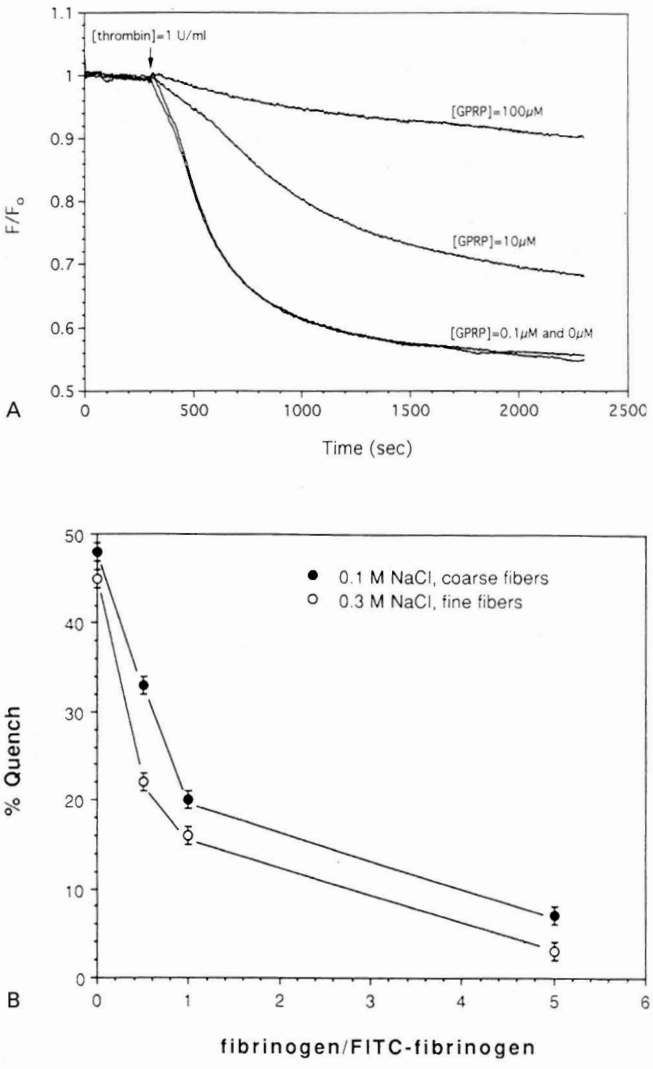
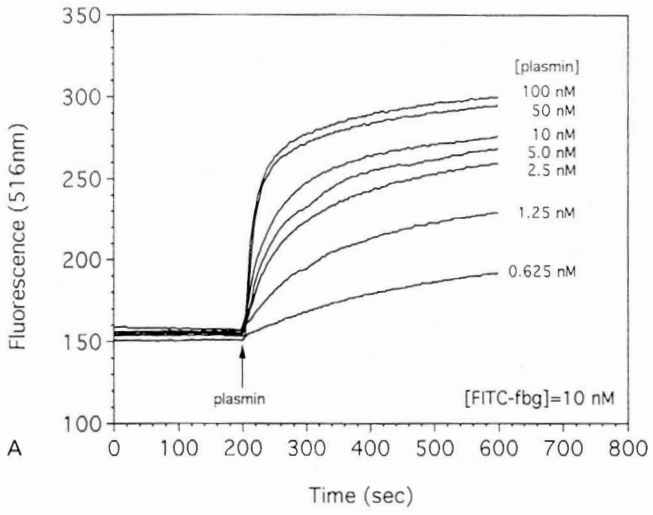
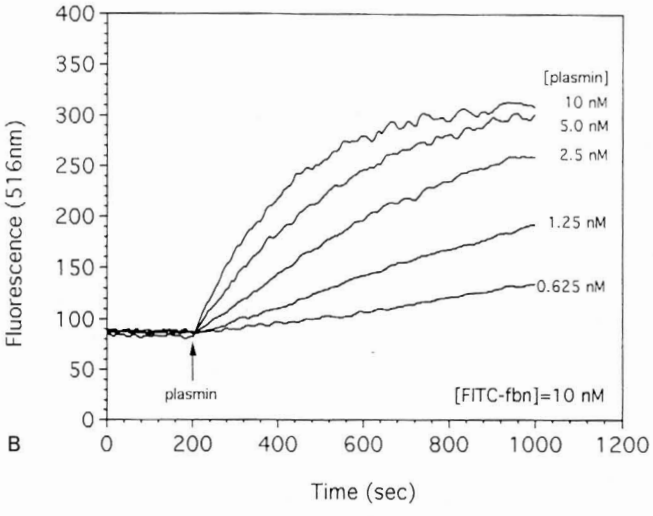


Fig. 2 Gly-Pro-Arg-Pro (GPRP) inhibited protofibril and fiber formation in a dose-dependent manner, resulting in a reduction in the rate and extent of quenching due to monomer-monomer interactions in the extending protofibrils (A). Thrombin (1 U/ml) was added to 80 nM FITC-fibrinogen in the presence of various concentrations of GPRP. The final extent of quenching produced by thrombin at 1 U/ml as a function of unlabeled fibrinogen to FITC-fibrinogen (35 nM) demonstrated the attenuation of monomer-monomer interactions in the polymerized fibrin (B). Using unlabeled fibrinogen in the polymerization reaction, a reduction in the final extent, but not the initial quenching rate, could be achieved by addition of various levels of unlabeled fibrinogen to reactions containing 35 nM FITC-fibrinogen in low (0.1 M NaCl, filled circles) or high (0.3 M NaCl, open circles) ionic medium

binding to its substrate was very rapid. Since the assay was most sensitive to fragment X generation – but relatively insensitive to subsequent lytic events such as fragment X to Y or fragment Y to D + E conversion – no accelerative phase was observed for fragment X generation from fibrinogen by plasmin. We found that the generation of terminal lysine residues did not appear to enhance the initial rate of fragment X generation. The initial rate of dequenching increased with increasing plasmin concentrations and saturated when the concentration of plasmin exceeded that of fibrinogen. The dequenching during fibrinolysis was similar to that observed for fibrinogenolysis, however the multiple modes of fluorescent probe interaction in the degrading fibrin fiber were not easily deconvoluted. We conducted SDS-PAGE analysis on





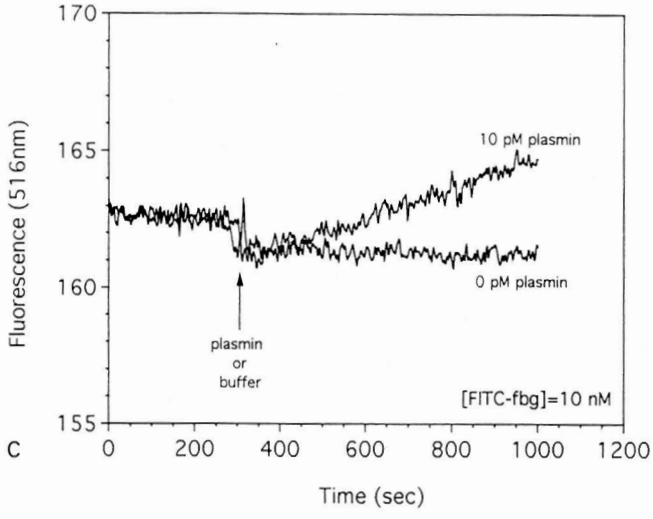
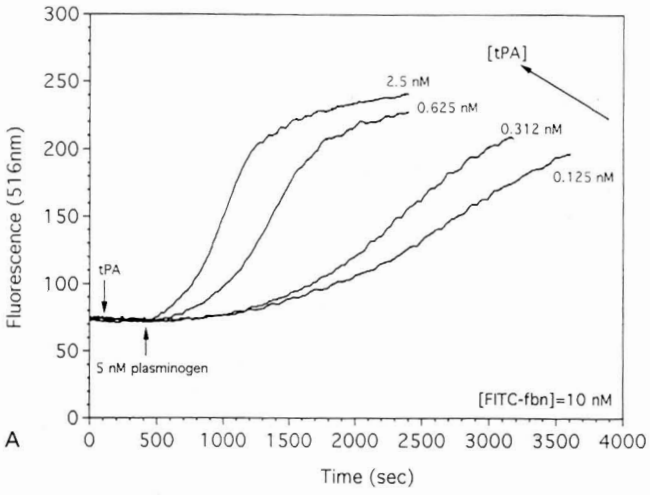


Fig. 3 Plasmin caused immediate and dose-dependent dequenching of FITC-fibrinogen (10 nM) as indicated by increase of fluorescence intensity at 516 nm (A). Plasmin caused a dose-dependent dequenching of FITC-fibrin (10 nM) (B). The addition of plasmin at a final concentration of 10 pM to FITC-fibrinogen (10 nM) caused a detectable rate of dequenching (C). A buffer control was used to correct for a slight dilution effect when reagents were added at 300 s. This experiment established the detection limit under the prescribed assay conditions, based on a criterion of a signal change that was over 3 times the noise level of about ±0.25 fluorescence units

partially degraded FITC-fibrin, and visualized fragments using epi-fluorescence microcopy of the gels. We found highly fluorescent bands which matched precisely the positions of fragment X, Y, D, and E when the gels were subsequently silver-stained. At the early stages of the reaction when most of the dequenching signal occurred, fragment X band was the most fluorescent and darkest silver-staining band.

We determined the detection limit using FITC-fibrinogen as a plasmin substrate under the typical assay conditions used previously. The addition of 10 pM of plasmin to fibrinogen caused significant dequenching rate that was well above the noise level (Fig. 3C) and could be detected within 300 s after initiation of the reaction. This detection of 10 pM of plasmin did not rely on signal smoothing, enhanced sampling data acquisition, or excessively long incubations over many hours to days. For the plasmin calibration curves (over a range of 0 to 10 nM plasmin) obtained at 200 s or 500 s after addition of plasmin into FITC-fibrin (Fig. 3B), the percent coefficient of variation ranged from 1.24% to 8.7% for determinations of plasmin concentration from 0.625 nM to 10 nM, respectively. Given the stability of the fluorescent baseline signal over many hours, obtainment of subpicomolar detection



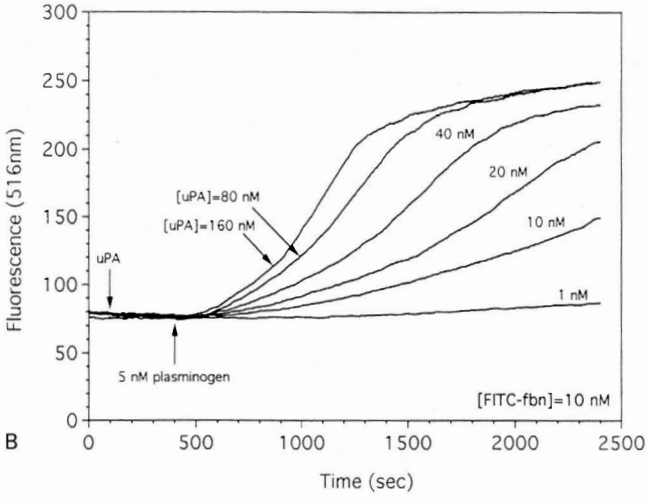


Fig. 4 Plasminogen activation assay based on plasmin-mediated dequenching of FITC-fibrin. Lytic reactions were initiated by addition of 5 nM of glu-plasminogen to 10 nM fibrin that had been preincubated with increasing concentrations of tPA (A) or uPA (B)

limits of active plasmin using a matched blank sample may be possible using longer incubation times and more advanced fluorimetric approaches.

We evaluated the utility of FITC-fibrin as a template for the assembly of tPA and glu-plasminogen. Activation of plasminogen would result in plasmin-mediated fibrinolysis of the substrate. We found for the assay of tPA that either FITC-fibrinogen or FITC-fibrin could serve as suitable substrates, but FITC-fibrin was a better substrate for ternary complex assembly and subsequent degradation. This is consistent with reports of tPA unique binding sites in intact fibrin, but not in intact fibrinogen (15). Subnanomolar levels of tPA (0.125 to 2.5 nM tPA) were easily detected within an incubation time under 500 s (Fig. 4A). In all cases, a lag phase was observed after addition of plasminogen activator to the reaction mixtures. The time of the lag phase was likely related to the times associated with plasminogen activation, carboxy terminal lysine residue generation, and enhancements due to conversion of glu-plasminogen to lys-plasminogen. For the tPA calibration curve obtained at 750 s or 1000 s after addition of tPA into plasminogen-containing FITC-fibrin (Fig. 4A), the percent coefficient variation was under 9% for determination of the tPA concentration at either time point. Levels of tPA above 5 nM caused a dose-dependent inhibition of the action of plasmin on fibrin (16). The sensitivity of the FITC-fibrin assay to measure tPA activity is in contrast to considerably less sensitivity of the assay for detection of uPA activity. Addition of 1 nM of uPA produced very little signal during a 2300 s incubation, while higher levels up to 160 nM uPA were needed to produce a rate of lysis comparable to that observed with only 2.5 nM of tPA (Fig. 4B). For the uPA calibration curve obtained 1000 s after initiation of the reaction (Fig. 4B), the percent coefficient variation ranged from 3.125% to 8.5% for determination of uPA concentrations from 10 nM to 80 nM. The sensitivity of the FITC-fibrin dequenching assay for detection of tPA was likely due to the ability of the substrate to assemble (and concentrate) tPA and plasminogen from very dilute solutions. The plasminogen activation rate expected for nanomolar levels of uPA and plasminogen would be expected to produce very low levels of plasmin in the time course of a 1 h incubation (17). Nonetheless, the sensitivity of the FITC-fibrin dequench assay was well beyond the sensitivity of chromogenic substrates such as S2251 for assay of tPA or uPA.

We conducted reactions to determine the detection limit of the assay for measuring plasminogen activator activity. We found that 20 pM of tPA could be detected in less than 20 min when added to 10 nM FITC-

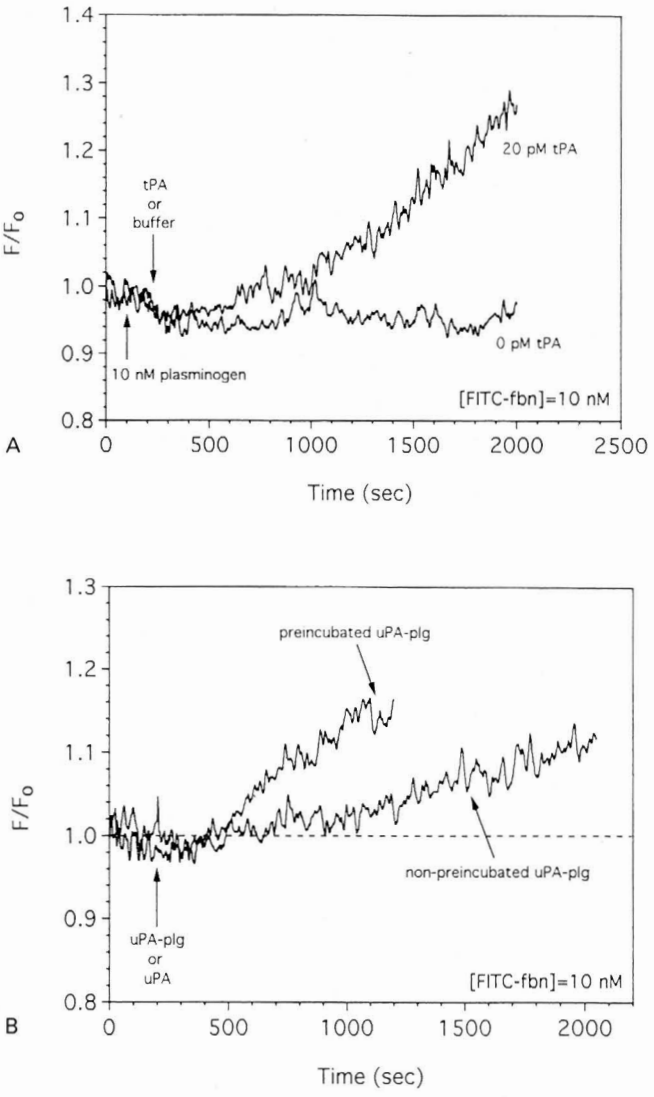


Fig. 5 The detection limit of tPA and uPA-mediated plasminogen activation was in the picomolar range. In a reaction mixture containing 10 nM FITC-fibrin and 10 nM glu-plasminogen, dequenching due to plasmin generation was detected when 20 pM of tPA was established (A). A buffer control was used to correct for a slight dilution effect when reagents were added at 300 s. Fluorescence intensity was normalized to initial intensity. In an experiment to establish the uPA detection limit (B), addition of 200 pM of uPA in the cuvette containing 10 nM FITC-fibrin and 5 nM glu-plasminogen generated a detectable signal due to plasmin-mediated dequenching (bottom curve). For comparison, when 24 nM of uPA was preincubated with 600 nM glu-plasminogen for 1 h at room temperature prior to addition to the FITC-fibrin (at final concentrations of 200 pM uPA and 5 nM glu-plasminogen), the plasmin activity was also detected

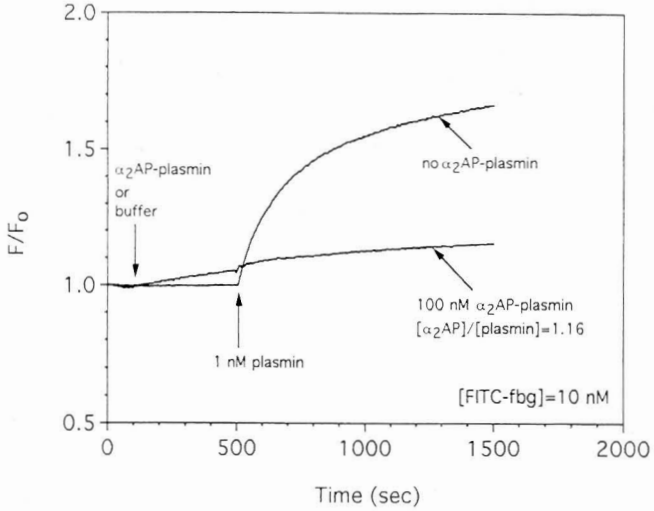


Fig. 6 Detection of plasmin activity in the presence of excess α_2-antiplasmin activity. A mixture of α_2-antiplasmin to glu-plasmin (1.16:1.0) containing 100 nM of complex was added to 10 nM FITC-fibrinogen. A small increase in fluorescence due to plasmin mediated degradation was observed. This plasmin was likely due to the dissociation of reversible α_2-antiplasmin-plasmin complexes. The excess activity of antiplasmin was demonstrated by subsequent addition of 1 nM of plasmin. The added plasmin had a short half-life in solution indicated by the small burst in dequenching that was rapidly stopped by the α_2-antiplasmin activity in solution. In comparison, addition of 1 nM of plasmin to FITC-fibrin in the absence of α_2-antiplasmin produced rapid and substantial dequenching

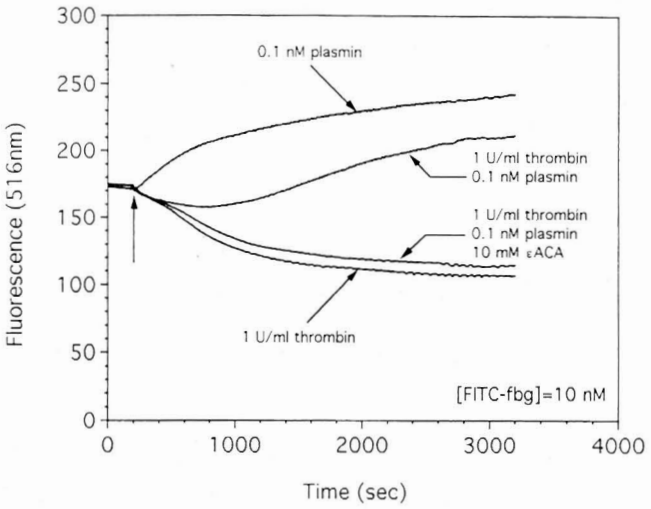


Fig. 7 A mixture of plasmin and thrombin produced a complex signal that displayed thrombin-mediated quenching followed by plasmin-mediated dequenching of the fibrin(ogen). The thrombin (1 U/ml) to plasmin (0.1 nM) ratio was chosen such that the time constants for polymerization and lysis were similar. Addition of a mixture of thrombin and plasmin with 10 mM ϵ-aminocaproic acid (ϵACA) blocked the activity of plasmin on FITC-fibrin(ogen) without effect on thrombin. This demonstrated that thrombin activity can be assayed in a sample containing plasmin activity. Additions of pure thrombin (1 U/ml) or plasmin (0.1 nM) are shown for comparison

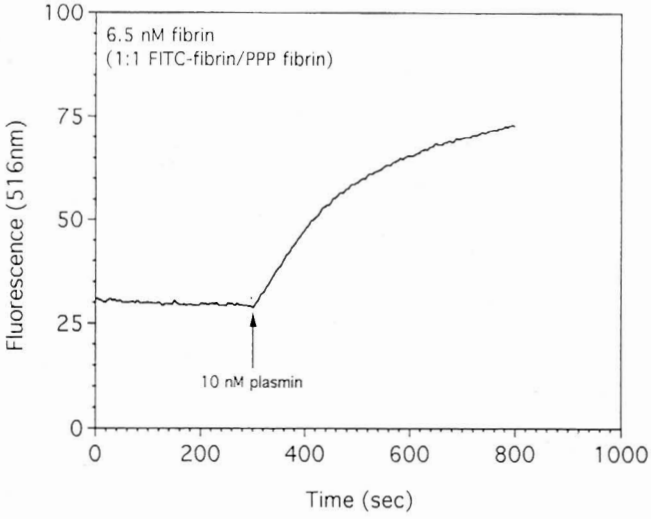


Fig. 8 Copolymerization of FITC-fibrinogen with platelet poor plasma (diluted to a similar fibrinogen level as the FITC-fibrinogen) produced a fluorescent suspension of fibrin. This suspension when treated with plasmin at 10 nM produced the characteristic dequenching due to fibrinolysis

fibrin and 10 nM plasminogen (Fig. 5A). Addition of 200 pM uPA to 5 nM plasminogen and 10 nM FITC-fibrin produced a significant dequenching detectable in less than 1 h after a 500 s lag time (Fig. 5B). In an attempt to enhance the detection limit of the assay for uPA, we preincubated (1 h at 22° C) a mixture of 24 nM uPA and 0.6 μM plasminogen in a 20 μl reaction volume in order to elevate the concentrations (and kinetics) for plasmin generation. We found some gain with this approach but still observed a lag time in dequenching of about 200 s after the reaction mixture had been added.

We have found that addition of α_2-antiplasmin to a reacting mixture of plasmin and FITC-fibrinogen will bring an end within 200 s to the fluorescence dequenching. Interestingly, we have also observed a slight plasmin activity in the presence of excess α_2-antiplasmin activity. When we added a preincubated 1.16:1.0 mixture of α_2-antiplasmin to plasmin (100 nM complex) to 10 nM FITC-fibrinogen, a small but significant rate of dequenching was apparent (Fig. 6). This dequenching was likely due to plasmin released from the dissociation of a reversible α_2-antiplasmin-plasmin complex. The excess activity of antiplasmin was demonstrated by subsequent addition of 1 nM of plasmin. The added plasmin has a short half-life in solution indicated by the short burst in dequenching that was soon abolished by the excess α_2-antiplasmin activity in solution. For comparison, addition of 1 nM of plasmin to FITC-fibrinogen in the absence of α_2-antiplasmin is shown in Fig. 6.

To study the use of FITC-fibrinogen as a substrate in the presence of more complex reaction mixtures, we added thrombin and plasmin simultaneously into FITC-fibrinogen suspension. A mixture of plasmin and thrombin produced a complex signal that displayed thrombin-mediated quenching followed by plasmin-mediated dequenching of the fibrin(ogen) (Fig. 7). The thrombin (1 U/ml) to plasmin (0.1 nM) ratio was chosen such that the time constants for polymerization and lysis were similar. Addition of a mixture of thrombin and plasmin with 10 mM ϵ-aminocaproic acid blocked the activity of plasmin on FITC-fibrin(ogen) without effect on thrombin. This demonstrated that thrombin activity can be assayed in a sample with plasmin activity. Reaction courses due to addition of pure thrombin or plasmin are shown for comparison in Fig. 7.

The reaction phenomena of lysis are vastly more complicated in whole blood or in plasma. We have sought to characterize the possibility of using FITC-fibrin as a probe that was incorporated in more complex fibrin polymerized from platelet poor plasma. Copolymerization of FITC-fibrinogen with platelet poor plasma (diluted to a similar fibrinogen level as the FITC-fibrinogen) produced a fluorescent suspension of fibrin. This suspension would be expected to contain α_2-antiplasmin, plasminogen, Factor XIII, thrombin, etc. incorporated into the fibrin fibers. When treated with 10 nM plasmin, this suspension produced the characteristic dequenching due to lysis (Fig. 8).

Discussion

Fibrinogen labeled with FITC was shown to be a useful fluorogenic substrate that allows the binding and assembly of blood proteins such as thrombin, plasminogen, plasmin, and tPA. FITC-fibrin(ogen) has a fluorescence emission that undergoes a 2- to 3-fold increase when degraded by plasmin. The increase in emission was due to loss of proximity-based quenching in the fibrinogen monomer or in the fibrin protofibril or fiber. When polymerized by thrombin, the fluorescence emission of FITC-fibrinogen decreases by about 50% due to increased probe interactions in the protofibril and fiber. Fluorogenic fibrinogen and fibrin present novel opportunities for kinetic studies under well mixed conditions on the fibrin(ogen) surface. Fibrin fibers are easily polymerized from dilute solutions of fibrinogen in a repeatable manner. Distinct from earlier studies which use fibrin suspensions to avoid diffusion artifacts in binding or kinetic studies (11, 18), the dilute polymerization of fibrin fibers does not involve sonication steps which may be difficult to standardize from one laboratory to another. Since the assay occurs under very dilute conditions using nM levels of substrate, over 10^5 determinations can be conducted from a preparation of 100 mg of FITC-fibrinogen which can be easily prepared in under 1 day and

used for at least 15 months. We observed that the assay was considerably more sensitive for measurement of tPA activity as compared to uPA. This was likely due to the ability of fibrinogen or fibrin to localize tPA and plasminogen in close proximity.

FITC-fibrin is also useful for the study of lytic events that occur on the surface of fibrin as opposed to those in solution. In the presence of ϵ-aminocaproic acid, plasmin generates no signal in the dequenching assay because it can not bind the substrate. However, the active site of the plasmin is still active under these conditions. During fibrinolysis, many different species compete for common sites or bind distinct sites (19-21) on fibrin. For example, plasmin and tPA can bind sites that exist in intact fibrin and to sites that exist in degraded fibrin. At any instant, the prevailing levels of antiplasmin, glu-plasminogen, lys-plasminogen, glu-plasmin, lys-plasmin, single-chain and two-chain tPA all contribute to the instantaneous rate of fibrinolysis. The ability to distinguish between fibrin-bound plasmin and unbound plasmin in a real time assay is facilitated by the use of FITC-fibrin.

Fluorescently labeled fibrin gels have been used before (22) in assays which measure release of fluorescent material into fluid surrounding the fibrin. However diffusion limitations of penetrating proteins into the fibrin are a problem in this format even with a well mixed extrinsic phase. Distinct from the approach of Genton (22), the present system takes advantages of molecular changes which occur in fibrin(ogen) as it is degraded. The assay occurs in a well mixed system devoid of diffusion limitations. Also, fluorescently labeled fibrinogen has been used in fluorescence polarization anisotropy assays (7). Yet this technique requires special polarization attachments which are not particularly rapid (typically giving 4 points per minute) and which are not suited for rapid assay of 96-well plates using fiber optics.

For kinetic studies of fibrinolysis and fragment X generation from fibrin or cross-linked fibrin, it may be possible to use fibrin containing about 1 to 5% of FITC-fibrin, such that fibril-fibril interactions in the fiber and monomer-monomer interactions in the protofibril are not contributing to the quenched state. The assay would then be most sensitive to fragment X generation and not sensitive to structural changes in the dissolving fibrin. These approaches may be useful for situations where FXIIIa-crosslinked fibrin is to be studied, without diffusion limitations often encountered with crosslinked gels.

Numerous possibilities exist for using fluorescently labeled fibrin suspensions in combination with other fluorogenic substrates for two or three color assays of multiple reaction kinetics. In future work, improved fluorescence labeling approaches that tag precise domains of the fibrinogen may provide more specific kinetic and mechanistic information. Since fibrinogen is a dimeric molecule, most labeling methods will attach at least two fluorescent molecules in close proximity with each other. The labeling method used in the present study is a simple, fast, and cost-effective approach to generate a substrate suitable for calibrated assays of various enzyme activities. Fluorogenic fibrinogen and fibrin take advantage of the unique structural and functional binding properties of intact fibrin and degraded fibrin. Possibilities also exist for generating reactions on cellular surfaces such as in incubations of platelets or endothelium in a manner to produce thrombin or plasmin activity which can be subsequently assayed by the techniques described in the present study.

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