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# Exposure of fibrinogen and thrombin to nitric oxide donor ProliNONOate affects fibrin clot properties

Christine C. Helms
*University of Richmond*, chelms@richmond.edu

Shannon Kapadia

Anne C. Gilmore

Zhexi Lu

Swati Basu

*See next page for additional authors*

**Authors**

Christine C. Helms, Shannon Kapadia, Anne C. Gilmore, Zhexi Lu, Swati Basu, and Daniel B. Kim-Shapiro

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Running Head: nitric oxide and fibrin clot properties

C. C. Helms[1]*, S. Kapadia[1], A. C. Gilmore[1], Z. Lu[1], S. Basu[2,3], D. B. Kim-Shapiro[2,3]

[1] Department of Physics, University of Richmond, Richmond, VA

[2] Department of Physics, Wake Forest University, Winston-Salem, NC

[3] Translational Science Center, Wake Forest University, Winston-Salem, NC

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* Correspondence: Christine Helms, Physics Department, University of Richmond,

Westhampton Way, Richmond, VA 23173

Tel: 804-289-8256; fax: 804-484-1542; E-mail: chelms@richmond.edu

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**Abstract**

**Background**

Fibrin fibers form the structural backbone of blood clots. The structural properties of fibrin clots are highly dependent on formation kinetics. Environmental factors such as protein concentration, pH, salt and protein modification, to name a few, can affect fiber kinetics through altered fibrinopeptide release, monomer association and/or lateral aggregation.

**Objective**

To determine the effect of thrombin and fibrinogen exposed to nitric oxide on fibrin clot properties.

**Methods**

ProliNONOate (5 µM) was added to fibrinogen and thrombin before clot initiation and immediately following the addition of thrombin to the fibrinogen solution.  Resulting fibrin fibers were probed  with an atomic force microscope to determine their diameter and extensibility and fibrin clots were analyzed for clot density using confocal microscopy. Fiber diameters were also determined by confocal microscopy and the rate of clot formation was recorded using UV-vis spectrophotometry. Protein oxidation and S-nitrosation was determined by UV-vis, ELISA and chemiluminescence.

**Results**

The addition of ProliNONOate to fibrinogen or thrombin resulted a change in clot structure. ProliNONOate exposure produced clots with lower fiber density, thicker fibers and increased time to maximum turbidity.  The effect of the exposure of nitric oxide to thrombin and fibrinogen were measured independently and indicated that each plays a role in altering clot properties.   We detected thrombin S-nitrosation and protein carbonyl formation after nitric oxide exposure.

**Conclusions**

Our study reveals a regulation of fibrin clot properties by nitric oxide exposure and suggests a role of peroxynitrite in oxidative modifications of the proteins. These results relate NO bioavailability and oxidative stress to altered clot properties.

**Introduction:**

Fibrin fibers are a major component in the structure of blood clots. They provide support to blood clots through the formation of a branching network of fibers. Fibrin fibers are formed from soluble fibrinogen after removal of fibrinopeptides A and B by the serine protease thrombin. Removal of fibrinopeptides A and B expose charged knobs which interact with oppositely charged holes on the distal region of a different fibrin monomer. This charge-charge interaction leads to protofibril formation and is followed by lateral association resulting in fiber formation and ultimately a branched fiber network. Additional covalent bonds are formed between fibrin molecules by transglutaminase, FXIIIa, after polymerization.

Fibrin network properties, both structural and mechanical, are influenced by polymerization conditions including thrombin concentration, fibrinogen concentration, methionine oxidation, pH, ionic strength, calcium concentration, and tyrosine nitration [1-9]. Structural changes at the single fiber and whole clot level affect the mechanical properties of fibrin clots. Li et al showed smaller fibrin fibers have a higher individual moduli than larger fibers and Ryan et al showed fibrin clot modulus varies with fiber density, fiber diameter, fiber length, and branch point density [9-11]. Structural and mechanical variations to the fibrin network alter the rate of clot fibrinolysis [12,13] and alter cell infiltration and cellular biochemical function [14]. Altered network properties have been linked to clotting disorders and diseases such as hemorrhaging, thrombosis, myocardial infarction, and stroke [11,15]. For example, myocardial infarction can be linked to an increased storage modulus, G' [9,15,16]. Altered clot architecture has been linked to diabetes, chronic heart failure and abdominal aortic aneurysms [17,18,18].

In this manuscript, we investigate the effect of nitric oxide (NO) exposure on fibrin clot properties. Suparaphysiological levels of nitric oxide are known to effect the activity of factor XIIIa [19] and therefore the crosslinking for fibrin fibers. Here we focus on the exposure of thrombin

and fibrinogen to NO through NO donor, ProliNONOate. This study  is motivated by the results of Davis et al. who reported a decrease in detection of NO produced by endothelial cells in the presence of fibrin and suggested this decrease in NO was due to a fibrin NO interaction [20].

The bioavailability and role of NO in cardiovascular signaling is a highly studied subject [21,22]. Although NO was originally thought to be short lived in the blood stream recent research suggests NO remains active in the blood stream through various mechanisms [23-26].  For example, the effect of NO metabolite, nitrite, on blood flow suggests the reduction of nitrite to NO in the blood stream [27].   In addition to the direct interaction between NO and fibrin clots suggested by Davis et al. byproducts of NO can interact with proteins and lead to modifications such as nitration, carbonyl formation and/or S-nitrosation.

Previous work looking at protein modifications has shown that fibrin tyrosine nitration increased clotting rate at low concentrations and decreased clotting rate at high concentrations [28]; while thrombin tyrosine nitration decreased thrombin activity [29].   The physiological relevance of these modifications is supported by measurements of S-nitrosation of plasma proteins such as albumin at physiological NO concentrations as well as shown implications of hypo or hyper S-nitrosation of blood proteins in disease states [22,30].  Similarly, studies reported protein nitration in many diseases such as cardiovascular diseases, Alzheimer's disease and Parkinson's disease [31-33]. Specifically nitrated fibrinogen is a risk factor in venous thromboembolism [34].

In this article, we investigated the role of in vitro nitric oxide exposure on fibrin clot properties and measured protein S-nitration, tyrosine nitration and carbonyl formation.  We determined NO exposure via ProliNONOate lead to these modifications.

**Methods**

All experiments were performed in fibrin buffer (pH 7.4, 10 mM HEPES, 5 mM $CaCl_2$, 140 mM NaCl).  Clots were formed from 1 mg/ml fibrinogen (Louisville APL Diagnostics, Doraville, GA, USA) mixed with fibrin buffer and 55 Lowey U/ml FXIIIa, where indicated, (Enzyme Research Laboratory, IN, USA) and clotting was initiated with the addition of thrombin (Enzyme Research Laboratory, USA).

Nitric oxide donor, ProliNONOate (Cayman Chemical, MI, USA) is stable in alkaline solutions but dissociates in a pH dependent manner.  Its half-life is 1.8 seconds at 37 ℃ in pH 7.4. Therefore, ProliNONOate was suspended in 0.01 NaOH at a stock concentration of 75 µM and was added in small volumes to the indicated protein solutions with a final concentration of 5 µM. ProliNONOate concentration was determined by absorbance at 252 nm.   The addition of ProliNONOate to the protein samples raised the pH of the samples from 7.4 to 7.5 and therefore controls were run with an equal volume of NaOH added to the sample to rule out any pH effect.

To determine the effect of ProliNONOate on fibrin clot properties, 5 µM ProliNONOate was added to fibrinogen prior to clot formation (Fibrinogen + NO), 5 µM ProliNONOate was added to thrombin prior to clot formation (Thrombin + NO) or 5 µM ProliNONOate was added to the clot solution immediately following the addition of thrombin and the solution was briefly mixed (All + NO).  Care was taken to perform this step quickly to avoid polymerization during mixing.

*Fiber density by confocal microscopy*

Two # 1.5 cover slides were coated with Sigmacote (Sigma-Aldrich, St. Louis, MO, USA) prior to clot formation.  Fibrin clots were prepared from 1 mg/ml fibrinogen, comprised of 10% Alexa 488-labelled fibrinogen (Invitrogen, Grand Island, NY, USA) and 90% unlabeled fibrinogen in fibrin buffer. Clot formation was initiated by the addition of 1 NIH unit/ml thrombin to the

fibrinogen solution.  ProliNONOate was added as indicated above. Immediately following the

addition of thrombin, the fibrin clot solution was placed on a coated cover slip inside a sealed

humidity chamber.  The clot was left to polymerize in the humidity chamber for 2 hours at room

temperature.  Coverslips were then sealed in holders to minimize drying of samples during

imaging.  Confocal images were obtained within 24 hours of clot initiation to ensure minimal

buffer evaporation using a Leica SP2 inverted confocal microscope with a 63X, 1.43 NA oil

immersion lens using a 488 nm Argon laser.  Regions of the clot were randomly selected and 30

slices of resolution 1024 x 1024 pixels were taken at an interval of 0.25 μm in z. Images were

obtained from three regions of each clot. A minimum of 12 clots were analyzed for each sample

condition.

Image J software (NIH) was used to analyze the images.  Fibrin fiber density of each images

stack were determined as previously described by Hugenholtz et al [35]. Briefly, lines were

placed across the slices at 10 μm increments and averaging number of fibers intersecting the

line were recorded. Fiber densities of three different regions per sample were averaged.

*Fibrin Fiber Diameter by AFM*

Fibrin fibers were formed on top of freshly cleaved mica by adding 0.5 NIH u/ml thrombin to 1

mg/ml fibrinogen and ProliNONOate as indicated. The fibers polymerized in a humidity

controlled environment for 2 hours before they were rinsed and air dried. The diameters of the

dried fibrin fibers were determined by AFM using AC or intermittent contact mode (Asylum MFP-

3D Bio AFM and MikroMash CSC37).  AFM scans were taken for a scan range of 5 μm x 5 μm

at a scan rate of 1 Hz and profile plots of line segments were used to determine fiber diameter.

Tip contact with the fibers during scanning can compress the fibrin fibers therefore reducing

fiber heights.  Similarly, the conical shape of the tip causes tip broadening effects increasing the

width of the fibers.  Therefore, we followed the procedure used by Domingues et al. and

measured the width of the fiber at half the maximum height to reduce error due to compression and tip broadening [36] (see Figure 2).    A minimum of 69 fibers were imaged for each sample condition.

*Fibrin Fiber Diameter by SEM*

In addition to AFM diameter measurement, we determined fibrin fiber diameters by scanning electron micrographs.  Clots were formed on Sigma Cote treated glass slides with 0.5 U/ml thrombin, 1mg/ml Fibrinogen, 55 units of Factor XIII and 5 µM ProliNONOate or NaOH as indicated. Samples formed in a humid chamber for a minimum of 2 hours before they were rinsed with HEPES buffer and fixed with a 2% gluteraldehyde solution for 30 minutes. The clots were rinsed and stained with a 1% osmium tetroxide solution for 15 minutes and rinsed again. Clots sat in dilutions of 50%, 80%, and 95% ethanol for 30 minutes each before being critical point dried on the Tousimis Samdri-795 Critical Point Dryer. The samples were sputter coated, mounted and imaged on the Jeol SEM at 10kV.

*Turbidity by UV-vis spectroscopy*

Clots were formed with 0.16 u/ml thrombin and 5 µM ProliNONOate, as indicated, in disposable micro UV-Cuvettes (BrandTech Scientific, Essex, CT).  Using a 10S Genesys UV-Vis Spectrophotometer (Thermo Scientific, MA, USA), absorbance was measured at 350 nm every 10 seconds for 1-2 hours. The measurements began immediately following the addition of thrombin.  We calculated the initial rate of fiber polymerization (OD/s during the first minute after the lag period), the clotting rate (OD/s at ½ max OD), and the clotting time (time at maximum slope) (n ≥ 5).  The first optical density measurement was used to baseline the data and the lag period was defined as the time before the OD increased beyond 0.05.

*Extensibility by AFM*

The ridged substrate was formed from Norland optical adhesive 81 (Norland Products, NJ, USA) using soft lithography and a PDMS stamp as previously described [37,38]. Fibrin fibers were formed on top of the 12 micron wide ridges by adding 0.5 NIH u/ml thrombin and 5 µM ProliNONOate as indicated. The sample polymerized for 2 hours and then was rinsed with fibrinogen buffer to remove excess fibers. 24 nm carboxylated fluorescent beads (Life Technologies, Grand Island NY, USA) were diluted in calcium free fibrinogen buffer and were nonspecifically added to the sample.  Fibers were visualized using an inverted fluorescence microscope (Zeiss, Thornwood NY, USA). Fibers were manipulated using an MFP-3D Bio AFM (Asylum Research, Santa Barbara CA, USA) and controlled step script allowing cantilever manipulation in the x, y, and z directions. Only fibers that were aligned perpendicular to or approximately perpendicular to the ridges were manipulated. An AFM cantilever was used to pull the fibrin fiber perpendicular to its orientation until it broke. To ensure that fibers were extended to their breaking points, samples that detached from the ridges were excluded.  The process was recorded using a CCD camera and Zen software (AxioCam MRm, Zeiss, USA). The initial and final lengths of the fibers were measured from the video images. The strain was calculated for each sample by dividing the change in length by the initial length.  The extensibility of at least 18 fibers were determined for each fiber formation condition.

*Protein S-nitrosation by ozone chemiluminescence*

5 µM ProliNONOate was added to 1 µM protein.  Protein S-nitrosation was measured by reductive chemiluminescence using the Nitric Oxide Analyzer (NOA), (GE Analytical Instruments, Boulder, CO).  Aliquots of sample were injected into the copper-cysteine assay, described previously [39], in the purge vessel of the NOA to measure the NO released.  The sample was treated with or without 5mM mercury chloride to ensure the signal was from a S-NO bond.

*Tyrosine Nitration by ELISA*

Nitrotyrosine concentrations were measured using a commercially available nitrotyrosine competitive ELISA (Cell Biolabs Inc, San Diego, CA). 1 mg/ml fibrinogen and 2 NIH u/ml thrombin were exposed to 5 µM ProliNONOate and were tested using the ELISA to quantify nitrotyrosine formation.   The ELISA was completed as indicated by the instructions.  Briefly, the samples and anti-nitrotyrosine antibody were added to the wells of a 96 well plate coated with nitrated BSA. After an incubation period the wells were rinsed and a horseradish peroxidase secondary antibody was added for colorimetric detection.   Absorbance measurements from the wells with the samples were compared to a nitrated BSA standard curve.

*Carbonyl detection*

Protein carbonyl concentration was determined by UV-vis spectrophotometry using a simplified alkaline 2,4-dinitrophenylhydrazine (DNPH, TCI America) assay.  Samples with 3 mg/ml fibrinogen or 20 µM Thrombin and 5 µM ProliNONOate or equal volume NaOH were prepared and mixed in equal parts with 10 mM DNPH.  DNPH reacted with ketones or aldehydes to form a derivative with an absorbance peak around 370 nm, similar to the absorbance of free DNPH. The maximum wavelength of the hydrazine derivative was shifted by the addition of 1.2 M NaOH and subsequent 10 minute incubation, as described by Mesquita et al [40]. Carbonyl concentrations were determined using a molar extinction coefficient of 22,308 $M^{-1}$ $cm^{-1}$ [40].

*Statistics*

Data is reported as the mean $\pm$ standard deviation unless indicated otherwise.  Statistical significance was determined by performing a two-tailed, homoscedastic student t-test and p-values are reported.

**Results**

We suspended ProliNONOate in NaOH for stability and prepared it at a high stock concentration to limit the amount of NaOH added to the samples during the addition of ProliNONOate. We added NaOH to the samples at a ratio of 1 part NaOH to 14 parts sample. The addition of NaOH to fibrin buffer at a ratio of 1:14 raised the pH from 7.4 to 7.5.  Therefore, we added NaOH to control samples at a volume equal to that of ProliNONOate addition to account for any effect of pH control.

Additionally, due to assay limitations the thrombin concentration changed between assays.  We indicated thrombin concentration for each assay in the methods section.  Therefore, one must take care when interpreting the results taken at various concentrations.

*Clot density*

Using confocal microscopy, we first tested for changes in clot density due to the addition of ProliNONOate.  The addition of 5 µM ProliNONOate immediately following the addition of thrombin to the fibrin clot solution (All + NO) decreased the fiber density of the clot compared to controls (Figure 1).  We noticed a by visual comparison a decrease in fiber density of the sample image compared to the control image and confirmed the decrease quantitatively by determining the average number of fibers intersecting lines drawn across the image. On average, there were $10 \pm 2$ fibers (average $\pm$ standard deviation) intersecting a 238 µm line passing through the NO + all sample while the control clot averaged $14 \pm 5$ fibers.  The decreased fiber density was significant ($p < 0.05$).

To determine if the effect was due to an interaction of ProliNONOate with thrombin or fibrinogen we tested ProliNONOate exposure to thrombin and fibrinogen independently.  The addition of 5 µM of ProliNONOate to thrombin, thrombin + NO, or fibrinogen, fibrinogen + NO, prior to clot formation significantly reduced the numbers of fibers intersecting a line to $9 \pm 2$ and $10 \pm 5$,

respectively (p < 0.01 and p < 0.05).  We measured a more significant effect of ProliNONOate addition to thrombin than to fibrinogen.   Therefore, ProliNONOate had an effect on clot density through its interaction with both fibrinogen and thrombin.

*Fiber Diameter*

A decrease in clot fiber density, may indicate an increase in fiber thickness. Therefore, we tested fibrin fiber diameter in clots in the presences and absence of ProliNONOate.  Using AFM, we measured the full width of the fibers at half their maximum height to minimize compression and tip broadening effects.  Additionally, we measured fiber diameters in clots imaged by SEM. Using both AFM and SEM, we measured an increase in fiber diameter under all conditions of ProliNONOate exposure (Figure 2).  ProliNONOate added immediately following thrombin addition significantly increased fiber diameter determined by AFM from the control diameter of 135 $\pm$ 34 nm to 161 $\pm$ 41 (p < 0.01).  We also measured a significant increase in fiber diameter when ProliNONOate was added to fibrinogen or thrombin independently.  The addition of ProliNONOate to thrombin increased the fiber diameter determined by AFM to 168 $\pm$ 43 (p < 0.01, compared to control) and the addition ProliNONOate to fibrinogen increased AFM determined fiber diameter to 155 $\pm$ 37 (p < 0.01, compared to control).

We saw the same significant increases in fiber diameter when diameters were determined by SEM.  Differences in the average diameters reported by the two techniques hint toward the independent limitations of the techniques. As mentioned above, the AFM requires contact during measurements and that contact can affect height and width measurements and likewise, sample dehydration needed for traditional SEM measurements can affect fiber diameters. However, the significant change in diameter was consistent through both techniques.   As before, we saw a larger change in diameter when ProliNONOate was added to thrombin. However, the addition of ProliNONOate to both proteins affected fiber diameters.

*Fiber formation kinetics*

The measured changes in clot fiber density and diameter suggest altered fibrin polymerization kinetics.   We measured the rate for fiber formation by UV-vis spectroscopy.  The addition of NO to thrombin before clot initiation significantly decreased the initial rate of clot formation from ($2.4 \pm 0.6$) x $10^{-3}$ OD/s to ($1.3 \pm 0.7$) x $10^{-3}$ OD/s and increased the clotting time from $62 \pm 20$ s to $77 \pm 30$ s ($p < 0.05$, Figure 3, Table 1).  The addition of NO to fibrin before clot formation trended towards a longer clotting time and a decreased initial rate of formation however; the results were not significant ($p > 0.05$, Table 1).

*Fiber Extensibilty*

Changes in protofibril lateral aggregation can affect clot density, fiber diameter and clot kinetics. Therefore, our data above support altered protofibril lateral aggregation.  The mechanism behind alter protofibril lateral aggregation may be related to differences in molecular interaction between protofibrils or due to the concentration of fibrin present in solution which can be altered by thrombin activity, thrombin concentration of fibrinogen concentration.

Fiber extensibility measurements may give some insight into altered molecular interactions between protofibrils.   The mechanism of the extraordinary extensibility fibrin fibers is not completely known.  Initial extensibility is thought to be due to extension of the coiled-coil region into beta sheets and unfolding of the globular gammaC domains [38,41,42].  However, the lengthening due to these changes only account for a small portion of the fibers extensibility. One hypothesis is that further extension comes from alphaC extension and protofibril sliding [38,41,42].  Therefore, the strength of protofibril interactions may affect fiber extensibility.

To test this hypothesis, we measured the extensibility of fibrin fibers using an AFM (data not shown).  We saw no change in extensibility when NO was added to clots during or prior to clot

initiation (p > 0.05). Fibers formed in the absence of NO had an extensibility of 182 % $\pm$ 36 %. Fibers formed when NO was added to thrombin or fibrinogen prior to clot formation or when NO was added immediately following the addition of thrombin to the fibrinogen solution had average extensibilities ranging from 158 % $\pm$ 59 % to 201 % $\pm$ 35 %, none of which were significant when compared to the control. The extensibility measurements agree with previously reported values for crosslinked fibrin fibers [43].

*Protein Modification*

To help explain the measured changes in clot fiber density, diameter and kinetics with the addition of ProliNONOate, we tested the samples for protein oxidation and S-nitrosation. We repeated the protein modification assays in duplicate and report a weighted average. Under the sample conditions we would not expect abundant formation protein S-nitrosation or oxidation products. Protein S-nitrosation requires the formation of a nitrosonium ion, $NO^+$, or transnitrosation from low molecular weight nitrosothiols such as S-nitrosocysteine [44] and oxidative products such as tyrosine nitration and carbonyl formation require superoxide and NO to form the species peroxinitrite.

As expected when we used an ozone chemiluminescence system to measure protein S-nitrosation, we detected low fibrinogen nitrosylation, 6 ± 3 mmole S-nitrosothiol per mole of protein. Interestingly, α-thrombin contained 90 ± 40 mmole S-nitrosothiol per mole of protein. Additionally, we used a competitive ELISA to measure protein tyrosine nitration and found 2 ± 2 mmole nitrated tyrosine per mole of fibrinogen and 160 ± 90 mmole nitrated tyrosine per mole of thrombin. The quantitative value of the nitration assay must be considered with some caution as the standard curve for tyrosine nitration used nitrated BSA and the affinity of the antibody for nitrated thrombin or fibrinogen may vary. Lastly we measured carbonyl formation using an alkaline DNPH assay. With the addition of 5 uM ProliNONOate carbonyl levels were below the

detection limit of the assay, however increasing ProliNONOate 10-fold, to 50 uM produced 880 ± 480 mmol of carbonyls per mole of fibrinogen and 390 ± 80 mmol of carbonyls per mole of thrombin.  This supports the assumption that carbonyls are produced through exposure of proteins to ProliNONOate.  A summary of the results of the protein modification assays are shown in Table 2. Thrombin has a larger amount of protein modifications in the tyrosine nitration and s-nitrosylation assays.  We believe this is due to the higher ProliNONOate concentration (5 $\mu$M) to protein ratio for these thrombin samples (2 NIH u/ml or 18 nM) compared to the corresponding fibrinogen samples (1 mg/ml or 2900 nM).

**Discussion**

The addition of NO donor ProliNONOate to the fibrin clot following the addition of thrombin led to changes in the resulting fibrin clot.  Fibrin clots formed in the presences of NO had a lower fiber density, larger fiber diameters and had a slower rate of fiber formation.  By testing the effect of ProliNONOate on fibrinogen and thrombin independently we found that ProliNONOate exposure altered clots through interaction with fibrinogen and/or thrombin.

Changes in structural properties, like those reported in our data, are often the result of altered formation kinetics which are due to changes in intermolecular interactions, thrombin activity or thrombin or fibrinogen concentration.  In regards to enzymatic activity, a decrease in thrombin activity is known to increase fibrin fiber diameter and decrease the fiber density of the clot [9,45,46]. In the presence of NO, we measured a decrease in the rate of polymerization, increase in fiber height by AFM and a decrease in fiber density of the clot, indicative of decreased thrombin activity. We measured 39 % carbonyl formation and 9 % S-nitrosation of thormbin.  De Cristofaro et al reported a decrease in the catalytic efficiency of thrombin with

oxidation [47] and Lundbald et al showed a decrease of thrombin enzymatic activity with oxidation [29]**.**

Additionally, we saw changes when NO was added directly to fibrinogen prior to clot initiation. The addition of NO to fibrinogen decreased clot density, increased fiber diameter and decreased the rate of clot formation.  The quantity of S-nitrosated and tyrosine nitrated fibrinogen after NO exposure was low with 6 mmol of S-nitrosothiols per mole of protein; however, we measured a carbonyl concentration of 100 mmol per mole of fibrinogen.  These data build on the changes in fibrin clot kinetics due to fibrinogen oxidation reported by Vadseth et al [3].

Vadseth et al. reported a decrease in the initial rate of fiber formation with 1.5 carbonyls per fibrinogen monomer [3].  This is consistent with our reported trend of a decrease in rate of clot formation. However, Vadseth et al. also reported an increase in the rate of clot formation at very low levels of tyrosine nitration, approximately 8 mmol of nitrotyrosines per mole of proteins [3] and data by Parastatidis et al.  measured an increase in the rate for fibrin formation, altered clot structure, increased fibrin stiffness and a decrease in clot lysis in the presence of fibrinogen nitration [2].

Similar fibrinogen tyrosine nitration levels in our samples to those of Vadseth did not lead to an increase rate of clot formation.  We believe this is due to a balance between fibrinogen tyrosine nitration and fibrinogen carbonyls. The significant but smaller effect on fiber kinetics, diameter, and fibrin clot density of fibrinogen exposure to ProliNONOate when compared to thrombin may be due to the competing mechanisms of carbonyls and fibrinogen nitration.

Our results suggest that exposure of fibrinogen and thrombin to ProliNONOate lead to the measured clot changes through protein oxidation which decreased the activity of thrombin and altered fibrin polymerization. However, the mechanism of oxidation upon NO exposure is unclear. A common pathway of protein oxidation requires the formation of peroxynitrite form NO and superoxide which should only exist in trace quantities in our solution. Therefore, we will focus future work on identifying the mechanism of oxidation to ensure the changes in clot structure are indeed due to oxidation and not another NO protein interaction.

NO is a small biological molecule produced by endothelial cells that line blood vessels. Its bioavailability is an active research area and varies substantially under disease conditions. Although the concentration of NO used in this study was high, elevated levels of tyrosine nitration has been measured in vivo [2,3].

Our data show NO exposure altered fibrin clot density, fiber diameter, and fiber formation kinetics. Measurement of protein oxidation suggest these changes are due to protein modifications. Previous studies report oxidative modifications to fibrinogen both increase and decrease the rate of fiber formation. He we show NO exposure leads to modifications that when considered cumulatively decrease the rate of fiber formation and consequently lead to associated structural changes.

**Addendum:**

S. Kapadia, A. C. Gilmore, Z. Lu, and S. Basu performed experiments, analyzed data, and drafted sections of the manuscript. C. Helms and D.B. Kim-Shapiro contributed to the experiment conception and design. C. Helms drafted the manuscript and supervised the research. All authors reviewed and approved the final manuscript.

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**Figure Captions**

**Figure 1.** Confocal images of (A) a control sample, (B) a sample with 5 µM ProliNONOate added at clot initiation (NO + All), (C) a sample with 5 µM ProliNONOate add to thrombin prior to clot initiation, (D) and a sample with 5 µM ProliNONOate add to fibrinogen prior to clot initiation. All images were taken under the same magnification. E) Lines spaced at 10 µm intervals were laid over the 238 x 238 µm images and the average number of fibers intersecting a line was calculated to determine fiber density of the clots. The graph displays the fiber density of clots formed under various conditions (average $\pm$ standard deviation, * $p < 0.05$, ** $p < 0.01$, n ranges from 13 to 20 clots).

**Figure 2.** Atomic force microscopy (AFM) analysis of dried fibrin fiber formed with 1 mg/ml fibrinogen, 0.5 NIH u/ml thrombin and 55 Lowey u/ml FXIII on top of cleaved mica.  Samples polymerize for two hours, after which they were rinsed with water and air dried. A)  A height map of an intermittent contact scan of a region of mica with dried fibers. B) Plot of height along the line segment indicated in A.  Fiber diameters were determined from such line segments.  The blue lines indicate the position at which the width at half the maximum height was determined. C) Bar graph of the average diameter of dry fibers formed under various conditions and imaged by AFM (error bars represent the standard deviation, * $p < 0.001$, n ≥ 40 fibers). D) Scanning electron micrograph of a fibrin clot.  We measured the diameters of fibers in the SEM images using Image J.  E) Bar graph of the average diameter of fibrin fibers formed under various conditions and imaged by SEM (error bars represent the standard deviation, * $p < 0.001$, n ≥ 23 150 fibers).

**Figure 3.** Spectrophotometer measurements of absorbance during fiber formation. Absorbance

measurements were taken every 10 seconds at 350 nm during fiber polymerization. A)

Absorbance versus time plots for control (black) and NO added to thrombin prior to clot

formation (gray).  The shadowing surrounding the solid lines indicates the standard deviation of

the measurements.   The control reaches its max OD around 3600 s and stops before the end

of the graph. The inset is an enlargement of the first two minutes of polymerization.   B)

Absorbance versus time plots for control (black) and NO added to fibrinogen prior to clot

formation (gray).  The shadowing surrounding the solid lines indicates the standard deviation of the

measurements.  For reference, NO added to thrombin prior to clot formation is indicated by a
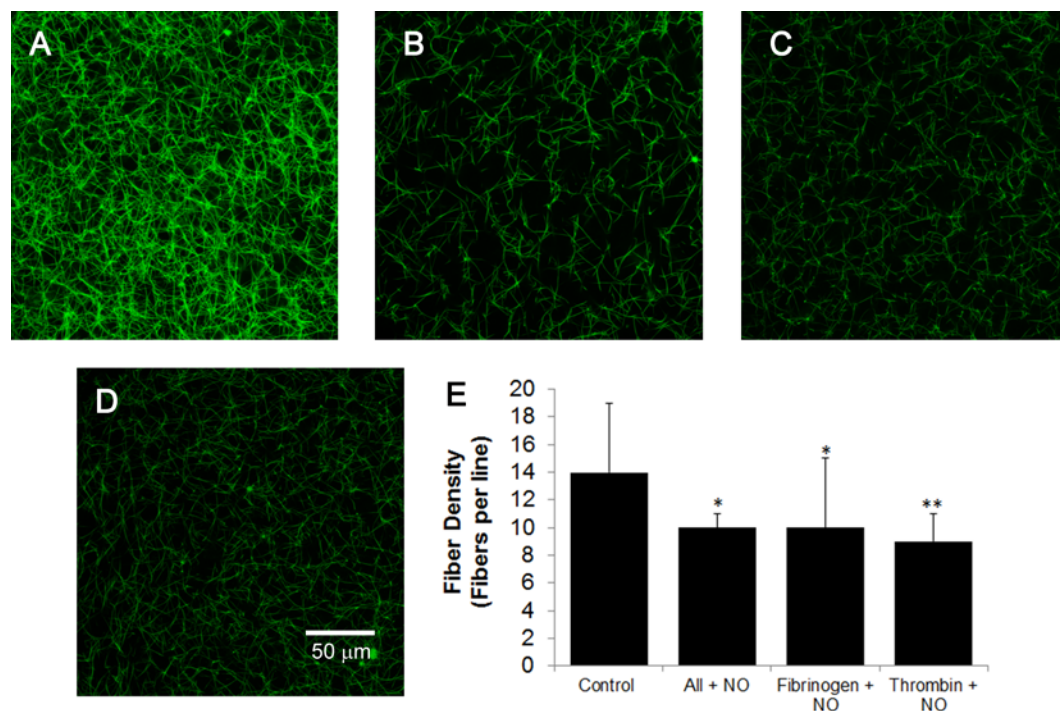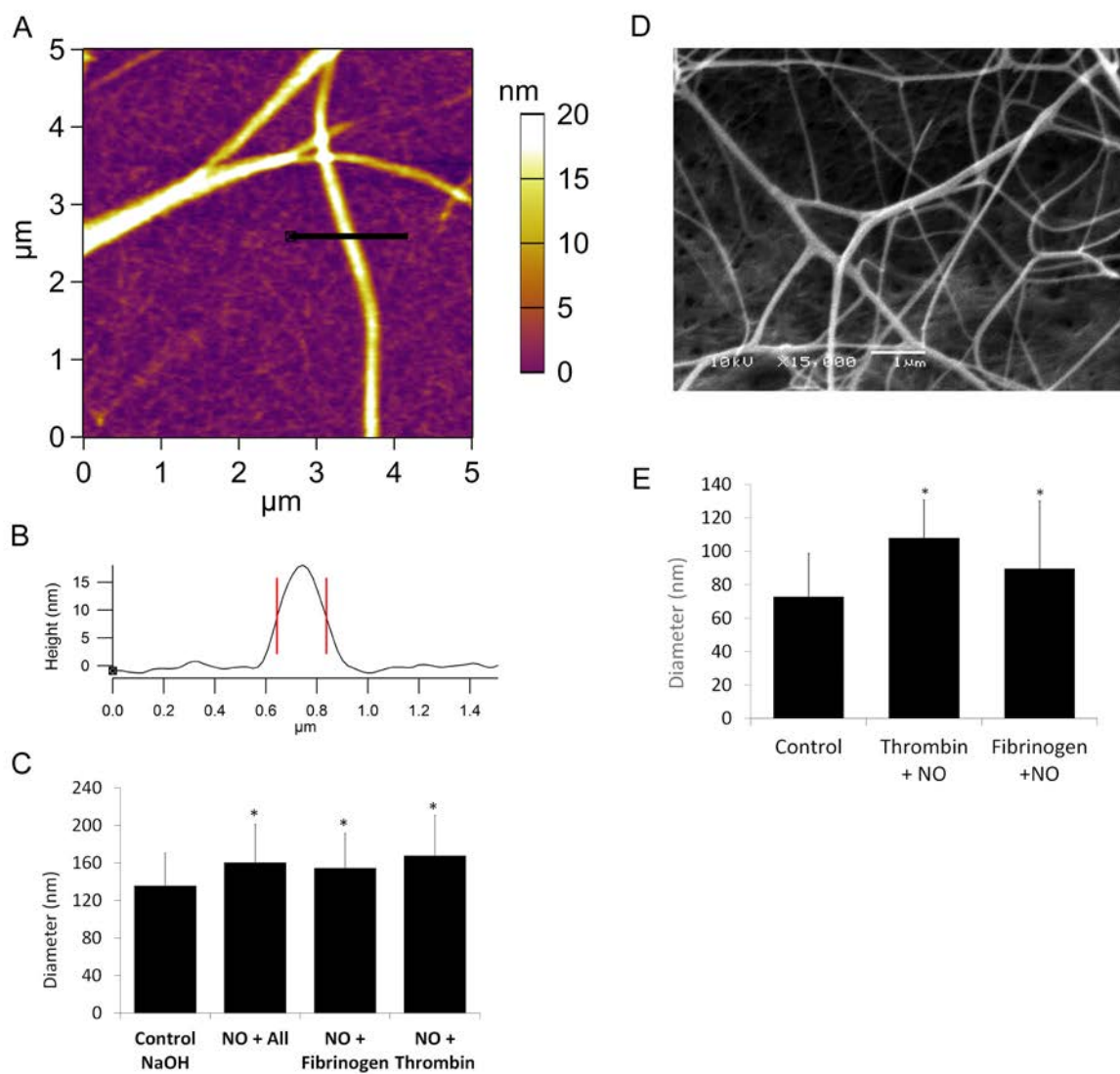
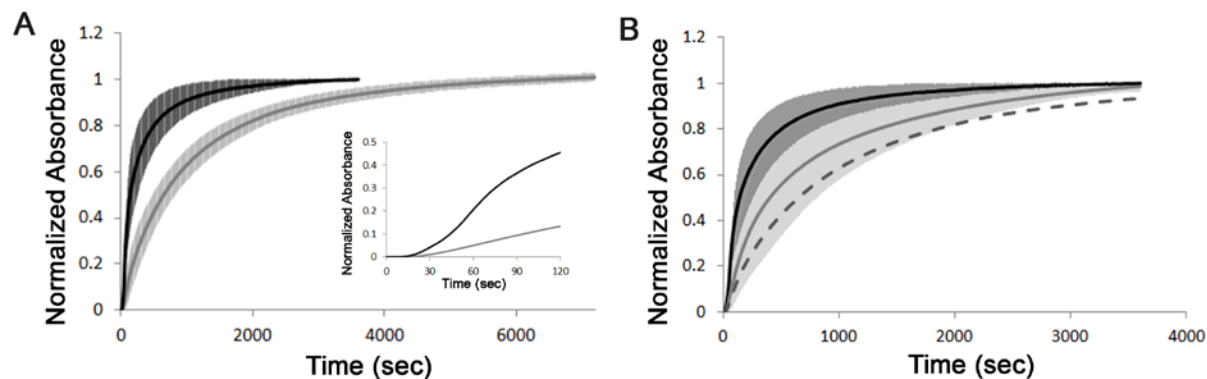dashed black line.

Figure 1

Figure 2

Figure 3



**Table 1** Turbidity measurements

|  | Initial rate (OD/s) | Rate at ½ max OD (OD/s) | Clotting time (s) |
|---|---|---|---|
| **Control** | $0.0024 \pm 0.0007$ | $0.0015 \pm 0.0012$ | $62 \pm 20$ |
| **Thrombin + NO** | $0.0013 \pm 0.0009^{\dagger}$ | $0.0004 \pm 0.0001^{\dagger}$ | $77 \pm 30$ |
| **Fibrinogen + NO** | $0.0017 \pm 0.0010$ | $0.0008 \pm 0.0004$ | $80 \pm 35$ |

$^{\dagger}\, p < 0.05$ when compared to control

**Table 2** Protein modification

|  | Fibrinogen (mmol/mole protein) | Thrombin (mmol/mole protein) |
|---|---|---|
| **S-nitrosation** | $6 \pm 3$ | $90 \pm 40$ |
| **Tyrosine Nitration** | $2 \pm 2$ | $160 \pm 90$ |
| **Carbonyl** | $880 \pm 480^{*}$ | $390 \pm 80^{*}$ |

Data is the weighted average and weighted standard deviation
*50 µM ProliNONOate was used in this assay, 10-fold higher than the other assays. Data is the average and standard deviation