Georgia Institute of Technology School of

Biomedical Engineering

Altering Fibrin Structure and Polymerization Dynamics Using Unaltered and PEGylated Fibrin Knob Peptides

May 5, 2010

Undergraduate Research Option Thesis

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TABLE OF CONTENTS

ABSTRACT

The behavior of the fibrin network in the presence of unaltered and PEGylated fibrin knob peptides is unknown. Fibrinogen, the principle protein investigated, participates in the coagulation cascade during hemostasis. Once activated by thrombin, it is converted to fibrin, which catalyzes Factor XIII to create a covalently cross-linked network.[2] Fibrin knob peptides bind to the fibrin pockets and block polymerization, altering the mechanical properties of the resulting network.[4] This study focuses on visualizing and analyzing confocal microscopy images to coordinate fibrin structure in the presence of peptides during polymerization. Using self-prepared and treated PDMS microfluidic devices, the solutions are mixed, injected, and imaged in real-time. The data is evaluated based on the calculated gel area fractions from 3D-rendered confocal images. The growth rates and maximal area of the fibrin networks are quantified and compared among the image sets. Data is used to predict how peptides alter the structure, development, and rate of fibrin network formation. Qualitatively, the incorporation of peptides into the fibrin matrix provides thicker fibers at lower overall network densities as compared to the control. The results show that fibrin knob peptides decrease the extent of network growth and that PEGylated peptides enhance this effect. A further understanding of peptides' effects on the fibrin network structure will have a strong impact by leading to an enhancement of the biological activity of fibrin and expanding the scope of applications of the fibrin scaffold.[8]

INTRODUCTION

The aim of this study is to evaluate the fibrin network in the presence of unaltered and PEGylated fibrin knob peptides, the application of which can be used for fibrin glues to potentially reduce scar tissue following surgery. This investigation defines fibrin network structure through real-time confocal microscopy and develops an encompassing model with both quantitative and qualitative bases for observation. By altering the network dynamics, the extent of uses of the fibrin scaffold may be expanded beyond current applications, representing an entirely new domain of biological matrices. Specifically, many experiments and results have indicated the power of the PEG (poly-ethylene glycol) peptide for matrix inhibition, but to date, no sufficient results show the benefits of such a network for practical applications. The difficultly in developing an encompassing model is that experiments must be conducted in a consistent manner to ensure that all networks have the potential to form under the same conditions. This model must focus on standard fibrin polymerizations and create bounds to govern how variations from the incorporation of peptides alter network properties. Often, researchers focus on a particular modification that causes inhibition of fibrin formation, but fail to run controlled tests to indicate how or simply if the network would have formed without inhibition. Using the implemented methods, this encompassing model approach serves to offer direct comparison and feedback that have yet to be realized by the current knowledge base.

Fibrinogen, the principle protein to be investigated, is contained in the blood and participates in the coagulation cascade during hemostasis. Once activated by thrombin, fibrinogen is converted to fibrin, forming long non-covalently bonded fiber strands.[1] Known as the fibrin-stabilizing factor, Factor XIII is catalyzed in fibrin and activated in the presence of thrombin to create a covalently cross-linked fibrin network. It collects the fiber strands to form a

mesh that characterizes the fibrin matrix. The structural origins of the fibrin clot and the architecture of fibrin assembly in the presence of different concentrations of thrombin and Factor XIIIa have been well studied. Electron microscopy has been customarily used to assess fibrin clot formation, but the results are unrealistic due to the unnatural conditions and fixation of the clots.[2] Confocal microscopy has become more popular for cell imaging due to its unique ability to reject out of focus light and because it has software capabilities to build 3-D rendered models. It has been implemented in a few cases to make observations of fibrin matrix polymerizations.

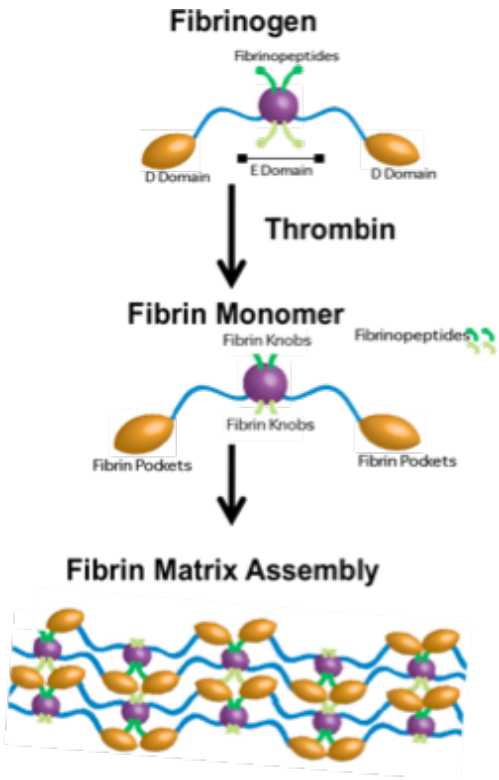


Figure 1: Fibrin Matrix

Fibrin knob peptides, specifically those with the initial sequence Gly-Pro-Arg-Pro, have been shown to modify fibrinogen.[3] These modifications are primarily observed by inhibition of cross-linking during polymerization. The Doolittle laboratory at UCSD was one of the first to publish work with the GPRP peptides. As reproduced in Figure 1, the research team showed that the fibrin knob peptides primarily bind to the fibrin pockets and block fibrin polymerization as a result of pocket interactions.[4] The peptide probes rely on the NH_2-terminal GPR on the fibrin α chain to inhibit network polymerization. This specificity of binding was further understood by examining the less-observed binding to neighboring sequences.[5] Early work with the influence of peptides on the fibrin network have served as a framework for future studies, yet major ambiguities exist in these findings that have yet to be answered. The mechanism of inhibition is relatively understood, but the advantages and direct comparisons with

uninhibited fibrin polymerization have yet to be presented. By using advanced imaging technology coupled with a unique set of data analyses and relationship models, this study fills many of the existing gaps of knowledge in fibrin polymerization dynamics.

By implementing a specific set these strategies, my goal is to develop a further understanding of fibrin network polymerization, using enhanced imaging and quantitative data to validate and expand many of the lightly understood mechanisms of fibrin formation. There exists a need to establish a relationship between uninhibited fibrin polymerization data and those supplemented with fibrin knob peptides. It is important, although often overlooked, to assess data for both mechanisms together and develop an encompassing model. It is believed that by understanding the fibrin network properties at the various conditions implemented, the architecture of the network may potentially be altered and reproduced as an enhanced network with material properties unmatched by standard fibrin polymerization observed under physiological conditions.

This investigation will begin with a focus on the methods developed after much trial and error. The results will be presented followed by a thorough analysis and explanation of findings. A complete model of peptide effects on the polymerization of fibrin is expected to correlate similarities and identify network changes along the way.

LITERATURE REVIEW

Chernysh et al. reportedly observed fibrin network formation using deconvolution microscopy. Deconvolution microscopy represents a viable approach to imaging the polymerization of fibrin, but for short time frames, confocal microscopy presents accurate and clean sample images without the computational errors introduced by deconvolution microscopy. Standard polymerization techniques are implemented here to observe real-time fibrin growth. The data obtained offers insight into the steps involved in fibrin polymerizations as well as a relative sequence of expected events. The results here focus solely on various imaging techniques of similar concentrations and conditions of the fibrin network. There is no inhibition or modification of the network.

The publications by Doolittle as discussed above provide key insight into the inhibition of Gly-Pro-Arg-Pro (GPAP) peptide strands that result from pocket interactions. Other researchers, such as Achyuthan, have investigated additional peptide strands, such as GPAP, to understand the inhibition exhibited by such a sequence. Achyuthan notes that GPAP "modifies the glutamine residues in the alpha- and gamma-chains of fibrinogen" causing "inhibition of transglutaminase cross-linking."[3] The analyses performed offer a beneficial understanding into the dynamic properties of the fibrin clot that prevent friendly coupling with foreign peptide strands. These results do not address ways to potentially use the modifications constructively.

In recent years, the fibrin network has been studied extensively for surgical applications including skin grafts and tissue-engineered skin replacements.[6] These fibrin gels rely on a high concentration of thrombin to be effective in making the clotting time as short as possible. These conditions are not favorable for the use of fibrin gel as an injectable scaffold. Some have studied the optimal conditions to use fibrin gel for cell delivery applications. Zhao found that fibrin gels

can be used to deliver fibroblasts for regeneration of the dermis tissue and that this can be applied to other tissue-delivery processes. An understanding of the factors influencing clotting time is also essential for this study. To implement optimal concentrations for fibrin gel applications, the fibrinogen concentration and the temperature are important factors. The gelation of fibrin is accelerated by increased temperature, nearly doubling at 25°C as opposed to 14°C. Under certain thrombin concentrations (~0.5 U mL^{-1}), the fibrinogen concentration serves as an independent factor for clotting time.[7]

The scope of literature available for this topic is both extensive and specific. Often, published research fails to address the initial question posed due to the discoveries and setbacks made along the way. To this end, most articles focus on one aspect of modifying fibrin networks in an attempt to indicate and present the mechanism of such inhibition. Doolittle, for instance, was the first to show that peptide strands can modify the fibrin network, and his results were based on the understood mechanism of standard fibrin formations. The problem with such a development is that under the conditions tested and implemented, no trials of standard networks were provided. This begs the question of whether the inhibition could have been based on the conditions and not on the peptide solutions implemented.

A study that conducts fibrin polymerization trials on with a large number of solutions and concentrations under the same conditions can provide direct comparisons among the networks. Without such a study, the present difficulties would remain and it would not be possible to develop a full understanding and determine all of the potential observations. Based on these conditional properties, it is important to establish a clear link between fibrin networks developed with and without the modification of peptides. This unanswered question remains essential in order to develop a more complete understanding of the material properties of fibrin.

Understanding the effects of peptides on fibrin network structure will have a strong impact by leading to a possible enhancement of the biological activity of fibrin.[8] Due to its unique, interlaced matrix, the fibrin network is one of the strongest naturally occurring biological matrices. Successfully altering fibrin network structure offers many experimental benefits and will broaden the scope of available applications of the network.

METHODS

The first step in running a micro-channel experiment was to make the devices as shown in Figure 2. A Sylgard solution consisting of a PDMS silicone elastomer base and its curing agent were stirred well in a plastic cup at a 10:1 ratio. The cup was then placed in a vacuum chamber to release all the air bubbles for about 1 hour. An aluminum-foil bowl was made, and a template wafer, made using photolithography, was placed into the bowl. Once the solution was free of air bubbles, it was poured onto the wafer in the bowl. The bowl was then covered with the top half of a large Petri dish to avoid dust accumulation and placed on a hotplate for ~ 5 hours at 60°C. The bowl was taken off the hotplate thereafter and allowed to cure and cool overnight. The next day, the solid silicon solution was peeled from the template wafer and the devices were cut out. Using a disposable needle or biopsy plunge, holes were bore into the devices at each of the ends of the channel. Compressed air was blown through the holes to ensure the path was clear of any debris. The devices were then cleaned with tape and placed in a large, labeled Petri dish for later use.

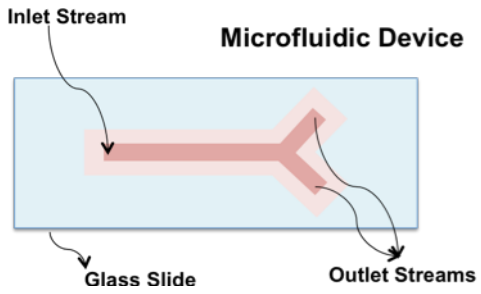


Figure 2: Microfluidic Channel Device

To prepare the micro-channel devices for an experiment, the desired number of devices were cleaned and set in a Petri dish with the channel facing upward along with a clean glass slide beside each device. The device and the glass slide were plasma-treated at 25 mA for 1:00 minute. Once the treatment was complete and the plasma chamber was purged, the Petri dish was removed and the device was flipped onto the glass slide ensuring even coverage. Using a 200µL pipetter, 1.5% 3-MPS in methanol was injected into the channel as a silanizer. The outlets were plugged at the ends and set for 20 minutes. After rinsing with ~300µL methanol, the devices

were injected with 0.5% gluteraldehyde in PBS (pH 7.4) and set again for 20 minutes, plugging the outlets. The devices were then rinsed with ~300µL dH_2O and allowed to incubate in a fibrinogen in HEPES (no $CaCl_2$) solution (Equation 1) for 30 minutes. The devices were then rinsed with ~300µL TBS + $CaCl_2$ and ready to be injected with the fibrinogen gel and imaged to assess network polymerization in real-time.

$$C_1V_1 = C_2V_2$$

$$\text{stock conc. fibrinogen} = 17.62\frac{mg}{mL}$$

$$\rightarrow \quad 500\mu L \text{ of } 0.1\frac{mg}{mL} \text{ fibrinogen soln. in HEPES buffer (no } CaCl_2)$$

$$\left(17.62\frac{mg}{mL}\right)V_1 = \left(0.10\frac{mg}{mL}\right)(0.5mL)$$

$$V_1 = 2.84\mu L \qquad (rest \Rightarrow \sim 497\mu L \text{ HEPES})$$

Equation 1: Making the 0.1 mg/mL fibrinogen in HEPES solution

ϕgn gels:

$$2 \text{ x } 250\mu\text{L gel @ } 2\frac{mg}{mL} \text{ with } 1\frac{mg}{mL} \ \phi gn \text{ labelled}$$

$$C_1V_1 = C_2V_2$$

$$\left(17.62\frac{mg}{mL}\right)V_1 = \left(1.8\frac{mg}{mL}\right)(125\mu L)$$

$$V_1 = 12.77\mu L\ \phi gn$$
$$+ 1\ aliquot\ labelled\ fgn\ (5.814\mu L)$$
$$+ 106.42\mu L\ TBS\ +\ CaCl_2$$

thrombin gel:

$$1\frac{U}{mL}\ thrombin \rightarrow 0.5\frac{U}{mL} \text{ overall in 250µL gel} \rightarrow \text{need } 1.25\mu L\ thrombin$$
$$+\ 123.75\mu L\ TBS\ +\ CaCl_2$$

thrombin + *FXIII* gel:

$$1\frac{U}{mL}\ thrombin \rightarrow 0.5\frac{U}{mL} \text{ overall in 250µL gel} \rightarrow \text{need } 1.25\mu L\ thrombin$$
$$1\frac{U}{mL}\ FXIII \rightarrow 0.5\frac{U}{mL} \text{ overall in 250µL gel} \rightarrow \text{need } 1.25\mu L\ FXIII$$
$$+\ 122.50\mu L\ TBS\ +\ CaCl_2$$

Equation 2: Standard Fibrinogen Polymerization Solutions

Peptide	Stock	3mM working (end vol 70µL)	TBS + $CaCl_2$
1-GPSPAAC	9.348mM	22.465µL	22.465µL
3-GPRPFPAC	5.924mM	35.45µL	34.55µL

ϕgn gels:

12.77µL *ϕgn*
+22.50 *peptide* working
+1 *aliquot labelled fgn* (5.814µL)
+83.91µL *TBS* + $CaCl_2$

thrombin gel:

$1\frac{U}{mL}$ *thrombin* → $0.5\frac{U}{mL}$ overall in 250µL gel → need 1.25µL *thrombin*
+ 123.75µL *TBS* + $CaCl_2$

thrombin + *FXIII* gel:

$1\frac{U}{mL}$ *thrombin* → $0.5\frac{U}{mL}$ overall in 250µL gel → need 1.25µL *thrombin*
$1\frac{U}{mL}$ *FXIII* → $0.5\frac{U}{mL}$ overall in 250µL gel → need 1.25µL *FXIII*
+ 122.50µL *TBS* + $CaCl_2$

Equation 3: Active Peptide Polymerization Solutions

Peptide	Molecular Weight (kDa)	Concentration (mg/mL)	Volume (µL) needed at 2:1 with fibrinogen in 250 µL gel
GPSP-PEG	5.775	4.813	9.09
GPRP-PEG	5.844	6.752	6.56

ϕgn gels:

12.77µL *ϕgn*
+ 9.09µL or 6.56µL peptide
+ 1 *aliquot labelled fgn* (5.814µL)
+ 97.33µL or 99.86µL *TBS* + $CaCl_2$

thrombin gel:

→ *same* as in *Equation* 3

thrombin + *FXIII gel*:

→ *same* as in *Equation* 3

Equation 4: PEGylated Peptide Polymerization Solutions

A number of polymerization solutions have been implemented and tested using human fibrinogen, fibrinogen labeled with ALEXA Fluorophore 555nm, human α-thrombin, Factor XIII, and the peptides GPRPFPAC, GPSPAAC, GPRP-PEG and GPSP-PEG (ERL, GenScript). All these substances were stored in the -80°C freezer prior to use. Equations 2 and 3 outline the standard make-up of these solutions. Deviations were taken from these specific amounts based on chemical conditions, starting concentrations, and desired end concentrations.

The process of imaging the devices for fibrin network growth over time involves the use of the ZEN LSM 510 Confocal Microscope. First, the system was initiated and ZEN 2008 imaging software was opened. The HeNe543 laser then was switched on and set for 555 nm labeled substances with the configuration of Ex 543 (Rhodamin, TRITC, CY3). The pinhole and gains were kept consistent with the system defaults, but these were adjusted if perhaps the pinhole was too large or the gain settings were not as desired. The relative position of the channel edge of the device was located using the ocular feature. Since observations were of network growth in real-time, it was important to finalize all settings prior to treating the device. The polymerization gel was then mixed by pulling up the thrombin with or without FXIIIa using a pipetter or needle and pumping up with it the labeled fibrinogen solution. The gel was injected into the device, upon which the timer was started. The device was immediately placed on the microscope, the channel position was verified, and a fast scan of the gel was performed to locate the glass slide. The first image in a z-stack of 15 images ($\leq$ 20 μm) was set about 15 μm above the position of the glass slide. A scan was completed with a time of 3.93 secs an averaging factor of 1 (~ 56 secs total). Preferably, the first image was taken at 1 minute, a feat that at times was difficult depending on the fluidity of the gel once injected and the system configurations, which sometimes need to be adjusted for consistency. Images were scanned every minute thereafter

based on the apparent intensity of the network with no movement in the x and y direction, but movement in the z-direction was necessary at times and noted. A 3D-rendered view was constructed using the ZEN 2008 software and appropriate thresholds were set based on density exposure and background noise. It was important to normalize area densities based on original, first scanned image, due to variations in transparencies and thresholds set by ZEN 2008. A single-plane image was then saved for each of the time intervals tested. The set of single-plane 3D-rendered images were then analyzed.

In order to observe the formation of the matrix in real-time within a suitable time frame for confocal microscopy, the implemented thrombin and Factor XIII concentrations have been tested and modified to obtain optimal growth within a 15 to 25 minute time frame. The formation of the initial fibrin scaffold followed by the expansion and growth of the network from these branch points represents the qualitative analysis of the data. Quantitatively, the area density of fibrin was calculated at each time interval using a program written in MATLAB. The densities were then normalized in Excel and plotted over time to show the rate and extent of the observed growth. Logistic best-fit lines, typically used for species growth under constrained bounds, were applied to the data to help determine a general model of fibrin growth.

Confocal and scanning electron microscope (SEM) image data were compared to verify and assess the confocal techniques and the effects on the fibrin network properties. An ultimate comparison will be made based on the formation and expansion of the fibrin network without peptide modification in the presence of thrombin and Factor XIII only. Due to the response of the fibrin matrix to any neighboring conditions and factors, consistency and repeatability of experiments was crucial and remains a priority.

RESULTS

A large number of images were collected based a series of pre-determined fibrinogen and thrombin plus Factor XIII solutions. Since the images were obtained with a variety of solution combinations and concentrations, both consistencies and questions of reproducibility were observed. For each set of concentrations and conditions, at least three experiments were performed and the best set was chosen for observations and analysis.

The first set of images (Figure 3) represents the control fibrin polymerizations with 0.5 U/mL thrombin and 0.5 U/mL thrombin + FXIII added respectively. These controlled image sets were essential is establishing a baseline of expected polymerization rates and final area densities. Figure 3A represents the fibrin network formed with the addition of thrombin only. It was observed that the channel remained fluidic during the first 6 minutes of imaging. At 8 minutes, the network scaffold first appeared, as fibers began to form and line up, followed by continued branched network growth and expansion. Growth was primarily outward from the branch points until about 14 minutes, at which point new stacks of fibers formed. An expansion of the network and incorporation of addition fibers was observed until about 20 minutes at which point imaging was completed. This network shows the passive individual fiber interactions as the fibers form and associate in bunches. When FXIII is added to the mix, as shown in Figure B, these neighborhood interactions become bound more tightly and, as expected, the area density of the network is much greater than with thrombin only. The network also formed much more quickly, exhibiting a nearly complete matrix at about 6 minutes. Thereafter, the matrix further expanded and increased in density as the mesh characterizations formed.

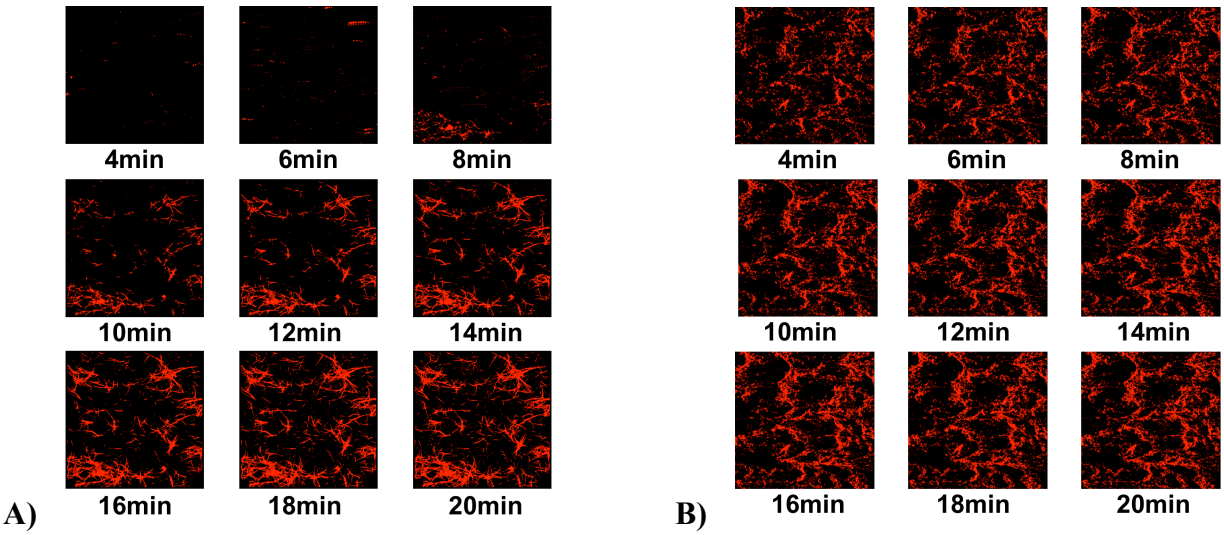


Figure 3: Real-Time Confocal Microscopy Images of control fibrin polymerizations. **A)** Fibrin network formed with 1mg/mL Fibrinogen + 0.5U/mL Thrombin. Polymerization was initiated after 6 minutes followed by steady network growth thereafter. **B)** Fibrin network formed with 1mg/mL Fibrinogen + 0.5U/mL Thrombin + 0.5 U/mL FXIIIa. Polymerization was rapid at first, providing an almost complete network within 6 minutes.

Implementing the MATLAB code as supplied in the addendum, the network area density for each image was calculated, and then plotted as a series in Excel. These plots, shown in Figure 4, indicate that FXIII has a much faster rate of network development initially, while with thrombin added on its own, the network requires time to form and lacks a pronounced density early on. These control plots serve as the bases for analysis of the inhibitory properties of the added peptides. In order to assess the extent of the specific peptide GPRP- inhibitors on the network, a series of control peptides were examined to determine their action on the matrix. These peptides, with the initial GPSP- sequence, were shown by Doolittle et. al. to have no effect on the thrombin-fibrinogen clotting time.[4] Therefore, the peptide GPSPAAC was used to verify the active peptides and GPSP-PEG was used to compare to the PEGylated peptides.

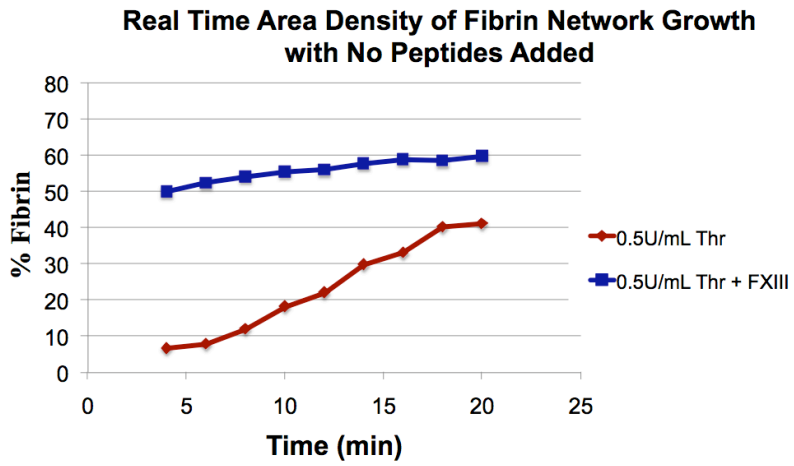


Figure 4: Control Fibrin Area Density Plots derived from MATLAB Image Analysis. The rate of area density growth for the network formed with thrombin only is greater than that with FXIIIa also added in. The implication of this observation stems from the rapid initial network growth occurring with the addition of FXIIIa, leaving little room for further polymerization.

The active, unaltered peptides were incorporated in the fibrin matrix and imaged during formation in real-time as presented in Figures 5. These peptides were taken at 100 times molar excess as compared to fibrinogen and were incubated with the fibrinogen for 30 minutes prior to imaging to ensure complete an interaction between the groups. When the network was formed in the presence of thrombin only, it is observed that the network contained less fiber clusters and the associations observed in the control groups are not as pronounced here. As shown in Figure 5A, the network was able to form into a sufficient matrix, but the density of the fiber groups were reduced. With the addition of FXIII, although the images are masked by some background noise, it is apparent that the fibers are much shorter and although they are closer together, the contacts appear weak.

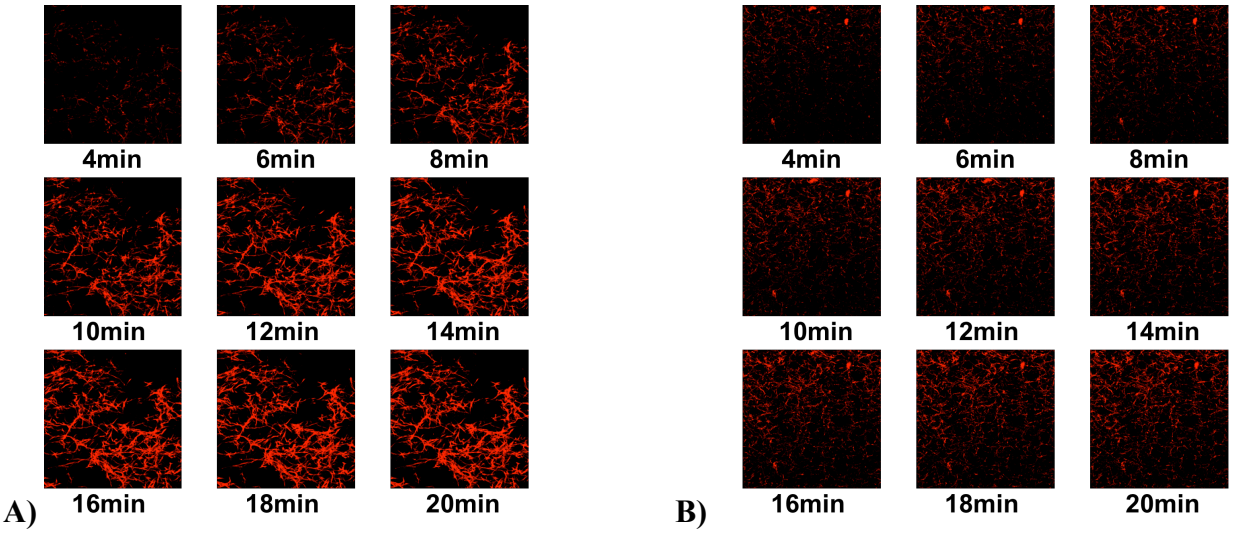


Figure 5: Real-Time Confocal Microscopy Images of fibrin polymerizations with the addition of active, unaltered peptides. A) Fibrin network formed with 1mg/mL Fibrinogen + 1:100 GPRPFPAC + 0.5U/mL Thrombin. Polymerization started at about 6 minutes and mimics the control network formation, but the fibers appear hindered from associating as tightly as in the control images. **B)** Fibrin network formed with 1mg/mL Fibrinogen + 1:100 GPRPFPAC + 0.5U/mL Thrombin + 0.5 U/mL FXIIIa. Background noise limits the quality of the image, but polymerization was again rapid at first due to the incorporation of FXIIIa.

An assessment of the fibrin area densities, plotted in Figure 6, show that the rates of polymerization, particularly with the addition of FXIIIa, have reduced as compared to the control areas. Whereas the control data showed an almost immediate network polymerization followed by slow growth thereafter, the incorporation of the peptide appears to have affected the action of FXIIIa, allowing it to follow the action of the thrombin only polymerization more closely. The network with FXIIIa did polymerize more rapidly at first, but close to equal levels were then reached with the network formed with no FXIIIa. The presence of the peptide is believed to have inhibited the action of FXIIIa to the extent of slowing down the time of polymerization. It's effect on thrombin remains unclear. The overall fibrin densities upon completed polymerization at 20 minutes shows a decrease in final area as compared to the control groups, with the associated qualitative reductions in fibrin interaction potentials.

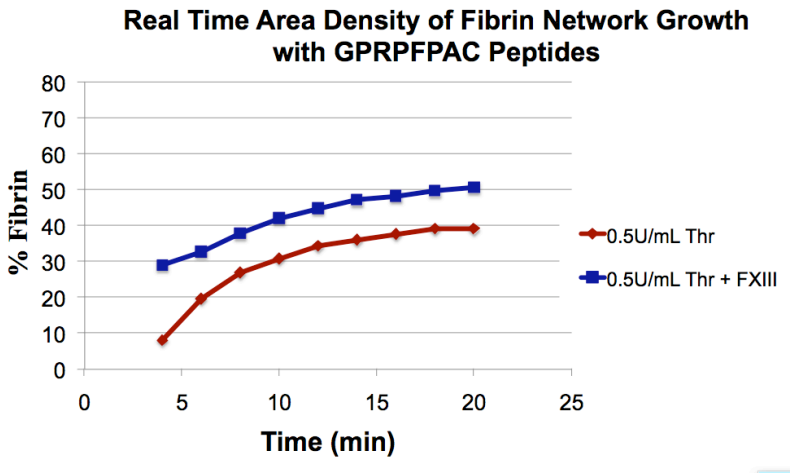


Figure 6: Fibrin Area Density Plots of Active Peptide supplemented fibrin network derived from MATLAB Image Analysis. The rates of growth for the networks closely follow similar trajectories. The area densities for the network formed with FXIIIa as well as thrombin shows increased area densities at all time points and is more rapid to show the dynamic network formation early on. Also, the final area density with FXIIIa is also greater than with thrombin alone.

The GPRPFPAC active peptides were modified with a 5 kDa PEG group to form the active PEGylated peptides GPRPFPAC-PEG. These peptides where then incorporated into the fibrinogen solutions to create polymerization networks as shown in Figure 7 once imaged in real-time under a confocal microscope. Here, the networks formed closely correlate with the expected matrix shape with and without FXIII, but the fibers are thin and not fully developed in series together. There exists some apparent background noise as well, whereby thresholds were set in the ZEN 2008 software to express as much of the formed fibrin network as possible. The modifications exhibited in the network from the addition of the active peptides are further enhanced here, leading to conformation changes in the fibrin assembly. In the thrombin only network, as in Figure 7A, these conformation changes appear in the reduction of branch points whereby the fibers line up side by side rather the cluster together. With the addition of FXIIIa, the fibers are much shorter and the extent of the associations within the network is reduced to local areas of fibrin collections. The increase in the spread of the available fibrin gel is observed here and any larger scale clusters are not able to form.

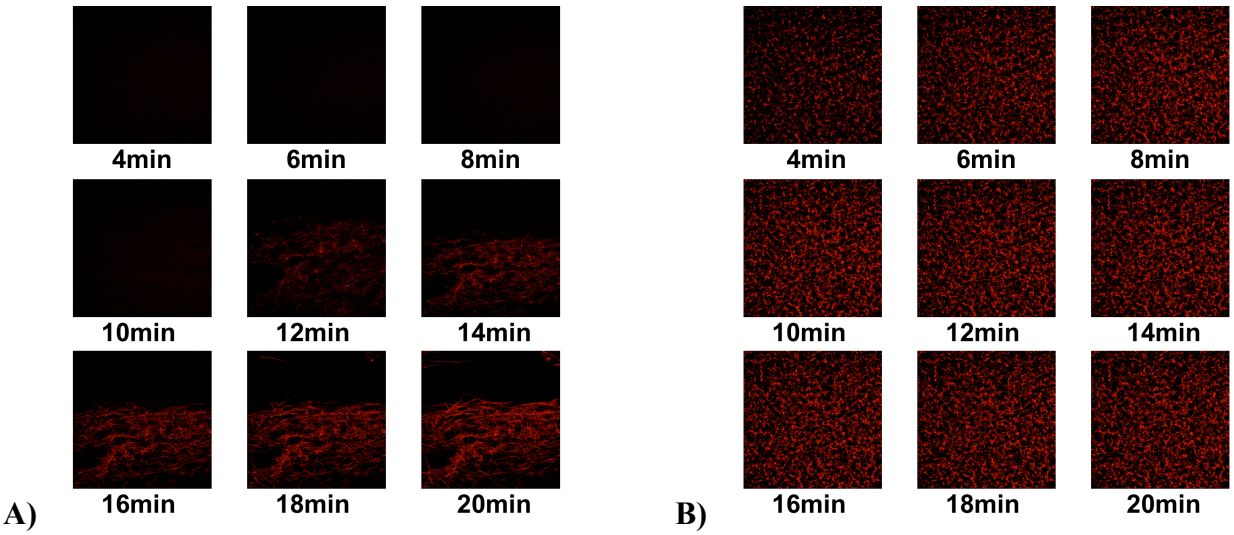


Figure 7: Real-Time Confocal Microscopy Images of fibrin polymerizations with the addition of Active, PEGylated peptides. **A)** Fibrin network formed with 1mg/mL Fibrinogen + 1:100 GPRPFPAC-PEG + 0.5U/mL Thrombin. Polymerization occurred at about 6 minutes, but the fibers are difficult to see due to image thresholding employed to reduce background noise. The network consists primarily of local fiber interactions. **B)** Fibrin network formed with 1mg/mL Fibrinogen + 1:100 GPRPFPAC-PEG + 0.5U/mL Thrombin + 0.5 U/mL FXIIIa. Small fibrin clusters constitute this matrix, whereby dense, more involved associations do not exist.

The area densities of the images, plotted in Figure 8, show a similar trend in polymerization rates that were exhibited by the active peptides. Here, the area density of the network with FXIIIa closely mimics that formed with no FXIIIa. This trend indicates that the PEG may be inhibiting the action of the FXIIIa to a greater extent than the active unaltered peptides. The high molecular weight of the PEG, polymerized in the presence of fibrin, is expected to enhance the inhibitory effect of the peptide sequence to which they are attached. In addition, since the both final fibrin network area densities are close, this inhibition property is further supported. Although it is unclear exactly how, the mechanical properties of the network at such a low final area density are also thought to be affected by the PEGylated peptides.

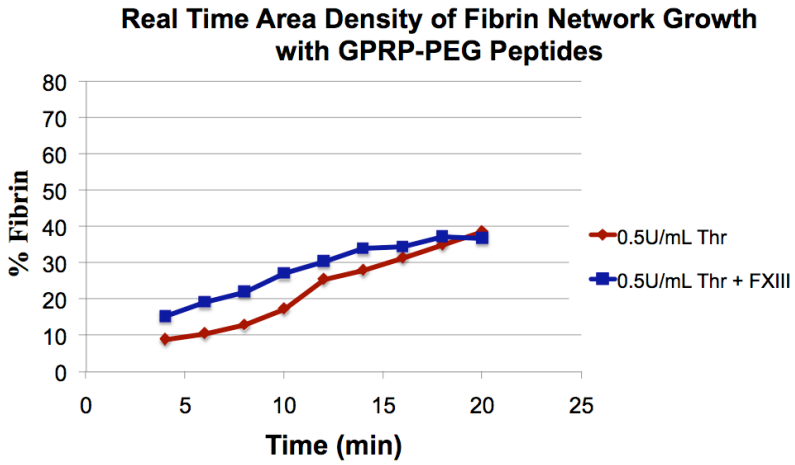


Figure 8: Fibrin Area Density Plots of Active PEGylated Peptide supplemented fibrin network derived from MATLAB Image Analysis. The rates of polymerization here are steady and close in both cases. The very low (< 40%) final area densities shown in this figure support the accelerated matrix inhibitory properties of the PEGylated peptides as compared to the unaltered active peptides.

DISCUSSION

The data show that the incorporation of peptides into fibrin gels for polymerization alters the network properties and the area fraction exhibited once mature. The application of such properties to fibrin glue has yet to be determined, but the behaviors of the fibrin network in the presence of peptides pose interesting questions about the resultant mechanical properties of the matrix. Due to the assessment of a number of different concentrations and conditions, it remains unclear whether the ones tested offer the optimal results for comparison. Further data collections and analysis are essential to fully identify the factors and observations to be accounted for.

Based on the assessment of the data, a logistic model, as shown in Figure 9, was chosen to fit the data and examine rate and maximum growth differences. This model was used as it is typically applied to species growth under constrained bounds. As indicated in the plot in Figure 9, the results of this model analysis show that fibrin knob peptides quantitatively decrease the extent of network growth. Unaltered active peptides with the initial GPR- sequence limit ultimate fibrin area density while exhibiting network polymerization at a faster rate. Moreover, PEGylated peptides enhance the effect of the fibrin knob peptides in the presence of Factor XIII by further lowering the observed area density, although polymerization rates remain similar to networks formed without the addition of peptides. These results represented ideal conditions whereby the networks all formed within a certain time frame and exhibited few extraneous variables in the setup. The rest of the data collected did not exhibit these trends leading to an assessment of reasons why and how to enhance future iterations of this study.

Logistic Models based on the form:

$$y = \frac{c}{(1 + ae^{-bx})}$$

Parameter Identification:

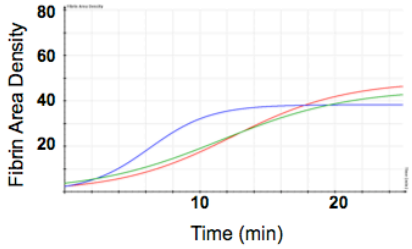
- a – scaling factor for exponential growth
- b – rate of area growth during polymerization
- c – represents the limiting value of the area density

Fibrin Polymerizations with Thrombin Only

Variable	a	b	c
No Peptide	19.75	0.24	48.62
GPRPFPAC	15.87	0.44	38.26
GPRP-PEG	11.31	0.21	45.23

Fibrin Polymerizations with Thrombin and Factor XIII

Variable	a	b	c
No Peptide	0.37	0.12	61.39
GPRPFPAC	1.83	0.20	52.41
GPRP-PEG	3.86	0.21	39.52



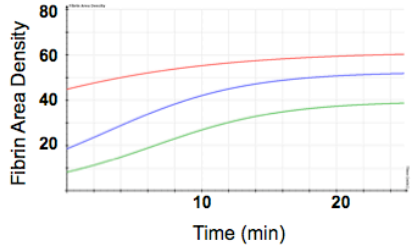


Figure 9: Logistic Model Analysis of the Optimal Polymerization Results. The logistic model assessed for the fibrin network polymerizations with and without FXIII show that Fibrin knob peptides decrease the extent of network growth. Unaltered active peptides with the initial GPR- sequence limit ultimate fibrin area density while exhibiting network polymerization at a faster rate. PEGylated peptides enhance the effect of the fibrin knob peptides in the presence of Factor XIII by further lowering the observed area density, although polymerization rates remain similar to networks formed without the addition of peptides.

Due to inconsistencies in much of the data (as shown in the addendum#1), this study proved inconclusive allowing for an evaluation of possible sources of error and ways to improve the methods of data collection and analysis. Sources of error may have first been apparent in creating the fibrin gels for polymerization. At some point, the study required a new batch of labeled fibrinogen, made under the same protocol originally implemented. It turned out that this labeled fibrinogen had a much lower binding affinity with the Alexa 555 fluorescence leading to a much less conjugated protein-fluorescence complex. Such an error led to the observation of more background noise in the image sets. Also, not all gels exhibited the same time of polymerization. This disparity was as large as 15 minutes long depending on the behavior of the network within the channel.

In addition, the amount of mixing of the gels prior to injection was variable and not subject to any predetermined protocol. The properties of the imaging software also presented some problem, namely that the pinhole size had to be adjusted based on the conditions of the device. Although this pinhole was usually set at the default 1 AU, this value was sometimes changed by necessity to obtain clear polymerization images. Further, this change led to variability in image gain intensities, which caused some image sets to be brighter, or at time more reflective than others. Lastly, software thresholding in MATLAB produced compounding error, whereby the threshold level for locating the fibrin matrix was depended on the previous adjustments and settings.

To improve this project for future iterations, it is proposed that a novel microfluidic technique be developed to limit mixing errors and maintain consistent results. The proposed model, as exhibited in Figure 10, will combine and mix the samples at the same time and is expected to improve the variations observed in the time of polymerization.

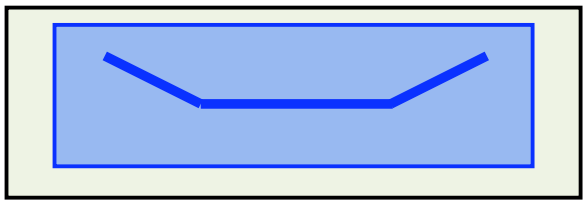


Figure 10: New Proposed Microfluidic Channel Device. The use of only one inlet stream and one outlet stream is expected to provide more even fibrin gel transfer ensuring the restriction of clumping and uneven mixing.

Using fibrin knob peptides to understand the polymerization dynamics of fibrin and alter network structure is an exciting and growing field of study. With advancements in imaging and mechanical properties machinery, researchers have the opportunity to see and understand biological functions and phenomena in ways that were never before accessible. Confocal microscopy, with its ability to focus and reject out of focus light as well as its built in 3D image stacks, provides a beneficial way to look at biological matrices. By observing real-time matrix

formation under a confocal microscope, qualitative observations may be made and compared to other imaging techniques. At this point, a large amount of images have been collected on the confocal and further analysis is desired for a more complete understanding of the behavior of the fibrin network under implemented conditions. Further, as proposed earlier, these images will be compared to images obtained from the scanning electron microscope, as shown in Figure 11.

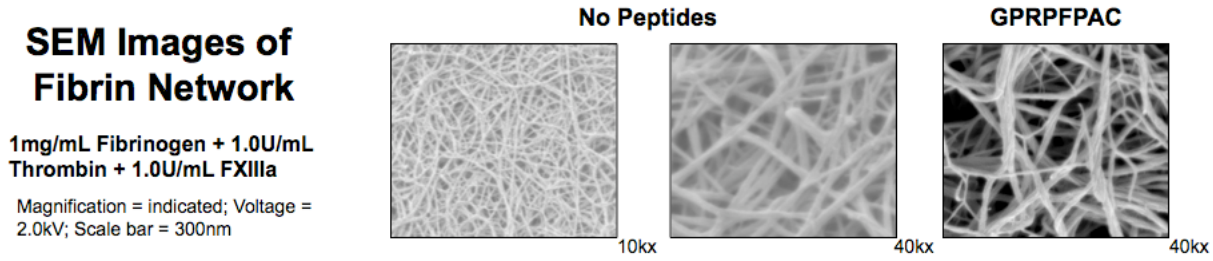


Figure 11: SEM Images of Fibrin Network at the end of Polymerization. These images with be compared with the final structure images obtained using the Confocal Microscope to correlate findings and establish a further understanding of the fibrin network properties in the presence of altered and unaltered peptides.

CONCLUSION

Although the inconsistencies in this study led to deviations that were unable to be controlled, the properties and characteristics associated with the selection of data provided in this paper give promise to the potential of future research. It is expected that the complications experiences here will provide a good basis for future iterations of this study in the ongoing quest to create an encompassing model and thereby develop a complete understanding of the behavior of the fibrin network in the presence of unaltered and PEGylated peptides. The imaging techniques employed here will be supplemented with new software capable of providing even more detailed representations of the fibrin network allowing for greater confidence in the data analysis techniques first presented here.

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6. Currie LJ, Sharpe JR, Martin R. The Use of Fibrin Glue in Skin Grafts and Tissue-Engineered Skin Replacements: A Review. Plastic and Reconstructive Surgery 108(6): 1713-1726, 2001.
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ADDENDUM

1. Inconsistent Image Sets showing Time, Color Intensity, and Density Variations

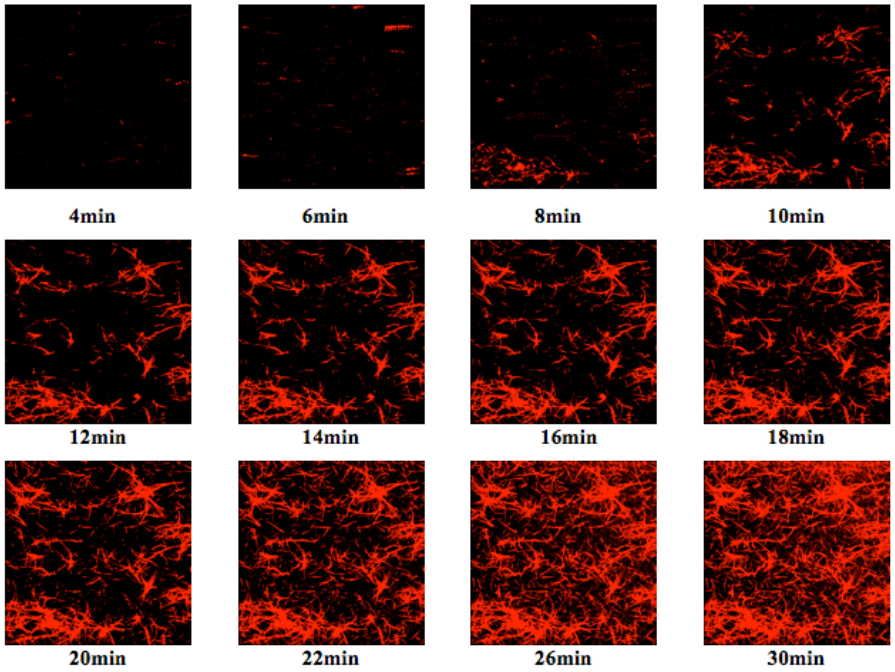


Figure 12: Polymerization with 0.5 U/mL thrombin

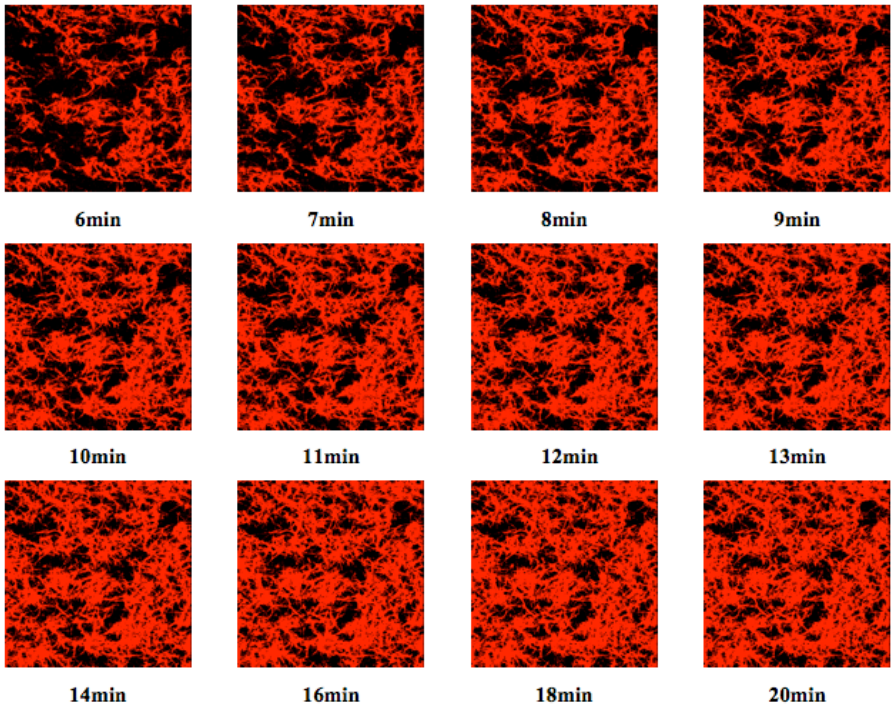


Figure 13: New Polymerization with 0.5 U/mL thrombin (2 X TBS + $CaCl_2$ concentration)

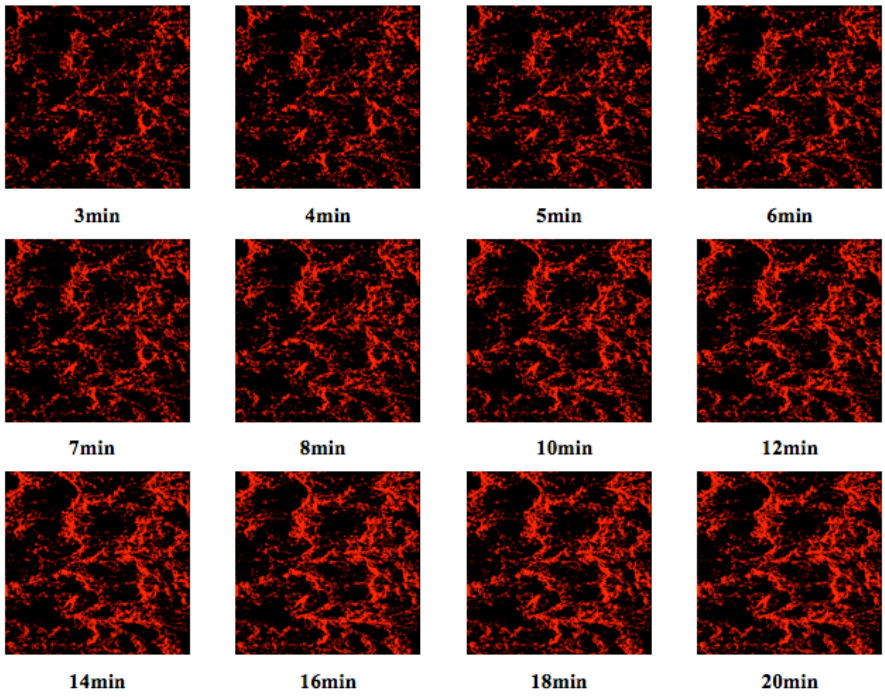


Figure 14: Polymerization#1 with 0.5 U/mL thrombin + FXIIIa (2 X TBS + $CaCl_2$ concentration, new FXIIIa)

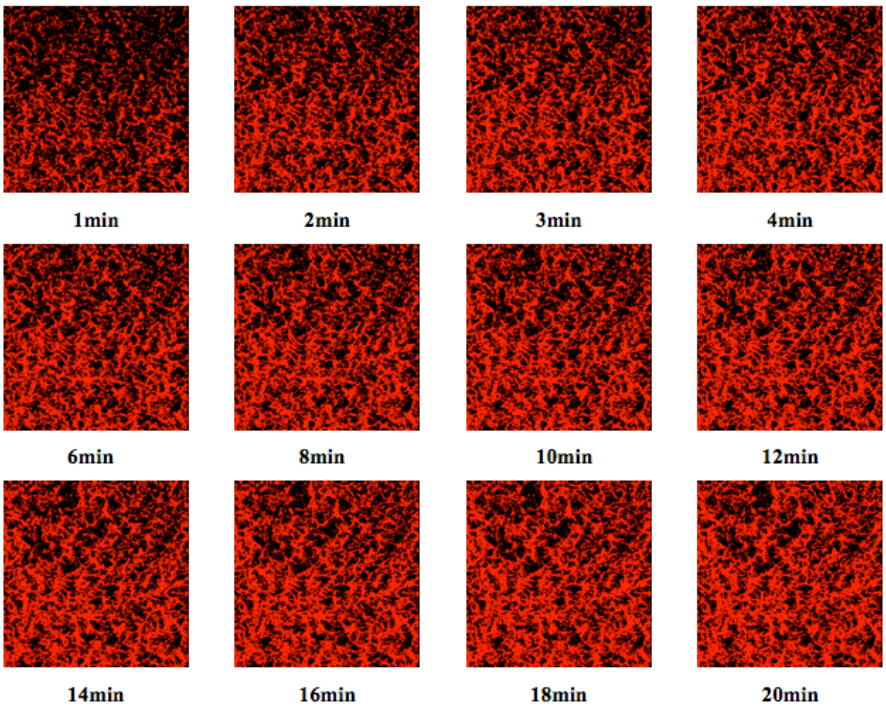


Figure 15: Polymerization#2 with 0.5 U/mL thrombin + FXIIIa (2 X TBS + $CaCl_2$ concentration, new FXIIIa)

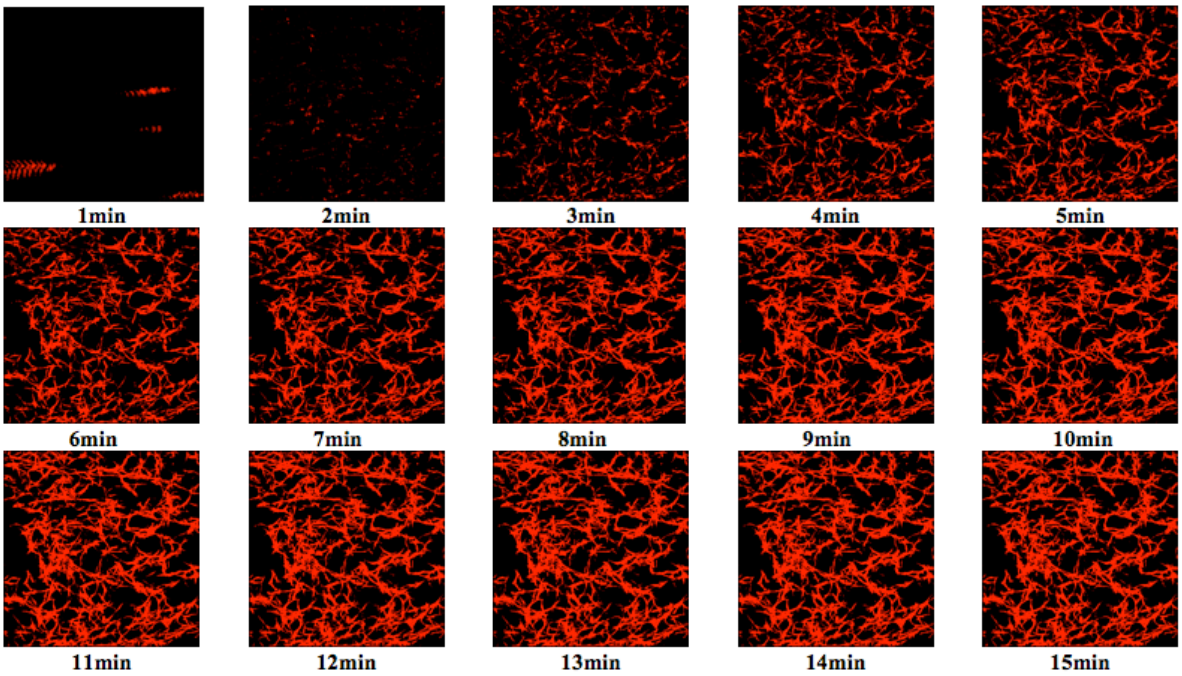


Figure 16: Polymerization with GPRPFPAC and 0.5 U/mL thrombin

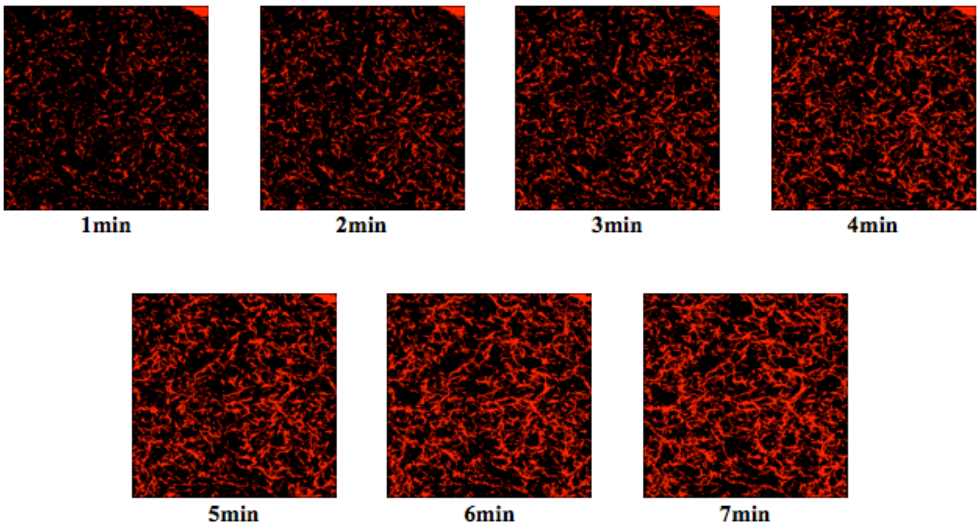


Figure 17: Polymerization with GPRPFPAC and 0.5 U/mL thrombin + FXIIIa

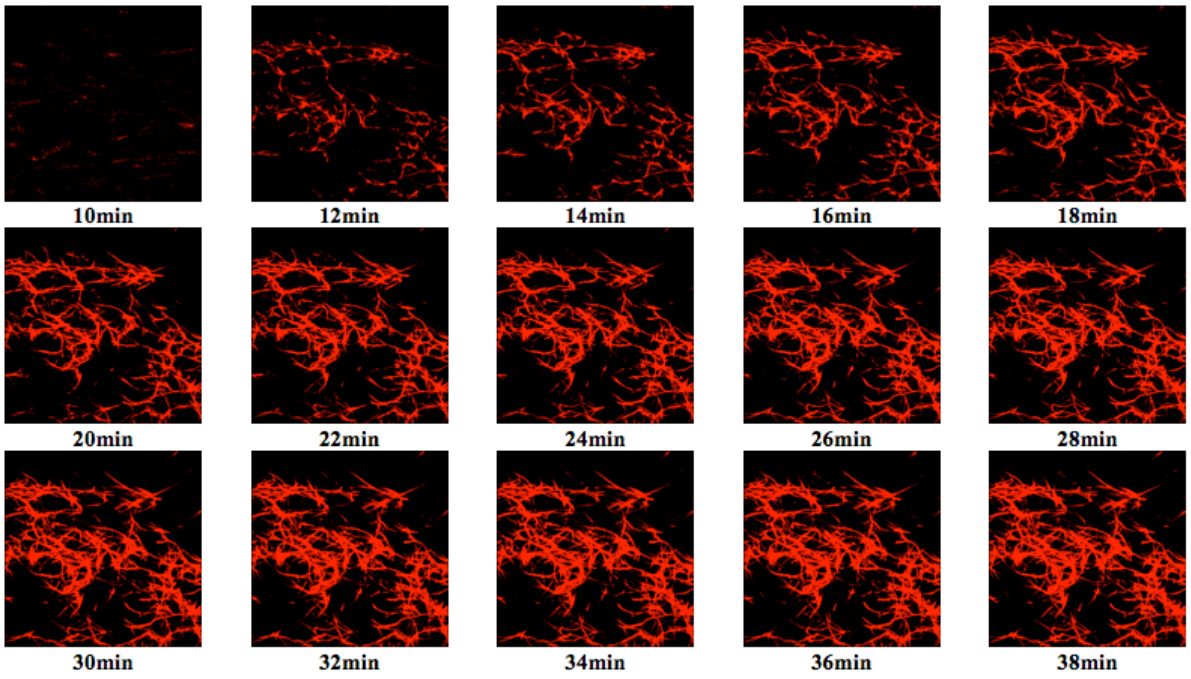


Figure 18: Polymerization with GPSPAAC and 0.5 U/mL thrombin

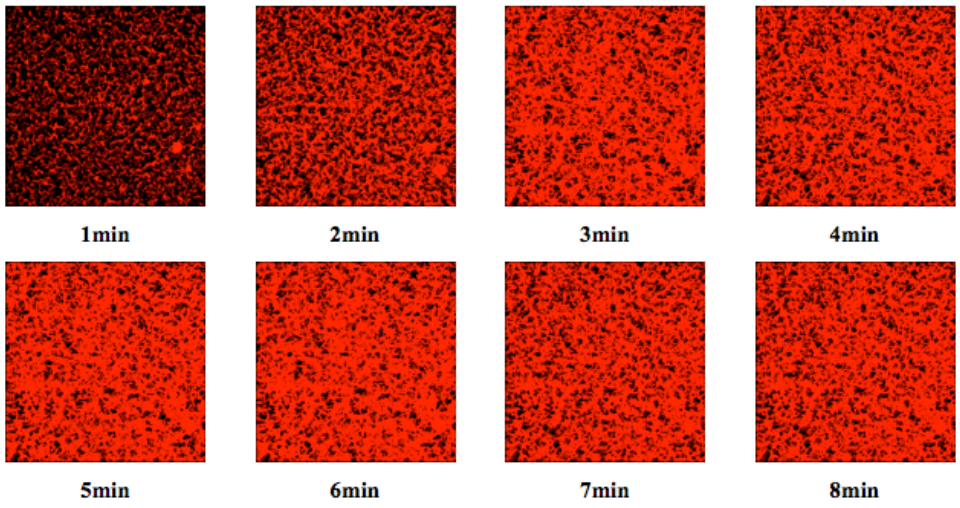


Figure 19: Polymerization with GPSPAAC and 0.5 U/mL thrombin + FXIIIa

2. MATLAB Image Analysis Code

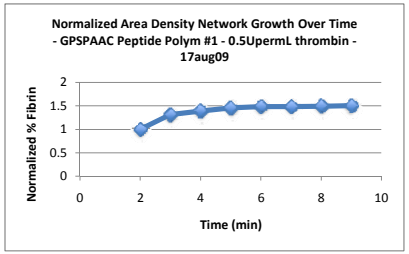
```
% % fibrin polymerization [date 00/00/0000]
% % Conditions: 0.5 UpermL thrombin + FXIII + Peptide
I = imread('4min_63x.tif'); %reads in the appr. image
crpr = 160; %crop coeff. for the image rows
crpc = 600; %crop coeff. for the image columns
[r,c,cm] = size(I); % r = 1064, c = 1948, cm=3
J = I(crpr:(r-crpr), crpc:(c-crpc)); %crops image by pixel indexing
[r1 c1] = size(J);
ncp = r1*c1; %returns total number of pixels in cropped image
% subplot(1,2,1); imshow(I); title('Original Image')
% subplot(1,2,2); imshow(J); title('B/W and Cropped Image')
isfibrin = (J >= 30); %0=black, so returns where in J is the image
% not black (has fibrin present)
y = sum(sum(isfibrin)); %sums up all ~=0 occurances
d = y/ncp; %ratio of non-black pixels to total pixels
fib_perc = d*100 %percent fibrin in image
fs = (142.58^2); tot_fibrin = d*fs; vol_fibrin = tot_fibrin*21;
%calculates other measurements based on overall z-stack thickness
```

3. Plots of all Peptide Supplemented Fibrin Network Data (refer to supplemental PDF document)

GPSPAAC peptide polymerization
0.5 UpermL thrombin

Trial 1

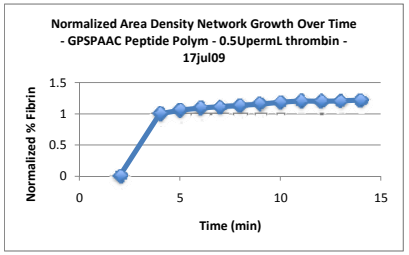
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	53.2787	1
3	69.9069	1.31209846
4	73.9823	1.38859056
5	77.6312	1.45707759
6	79.1387	1.4853722
7	79.2151	1.48680617
8	79.6323	1.49463669
9	80.128	1.5039406



(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 100X used
(4) easy to see strand by strand (standard network formation observed)
(5) steady growth rate hit plateau early
(6) no movement in z direction necessary until later in imaging

Trial 2

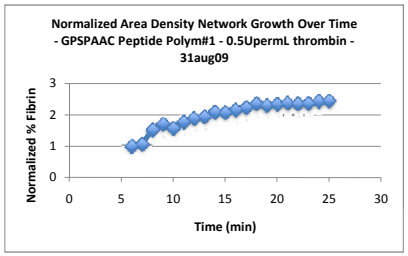
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	0.0228	0.00031518
4	72.3397	1
5	76.421	1.05641854
6	78.995	1.09200066
7	80.4453	1.11204912
8	82.1665	1.13584242
9	83.626	1.15601806
10	85.7449	1.18530904
11	87.2308	1.20584962
12	87.0928	1.20394196
13	87.3655	1.20771167
14	88.2937	1.2205428



(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 63X used
(4) more cluttered - fast but standard polymerization

Trial 3

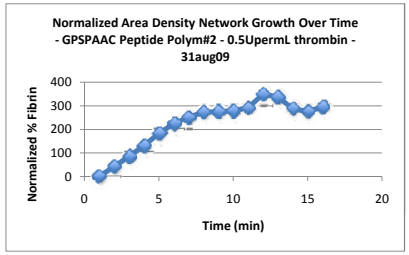
Time (min)	MATLAB percent fibrin	normalized percent fibrin
6	21.1575	1
7	22.4727	1.06216235
8	31.9718	1.51113317
9	35.788	1.69150419
10	33.1357	1.56614439
11	37.1712	1.75688054
12	39.5719	1.87034858
13	40.7242	1.92481153
14	43.758	2.06820276
15	43.4283	2.05261964
16	45.2338	2.13795581
17	46.7404	2.2091646
18	49.1806	2.32449959
19	48.1324	2.27495687
20	49.0551	2.31856788
21	49.6583	2.34707787
22	49.2644	2.32846036
23	49.5143	2.34027177
24	50.923	2.40685336
25	51.218	2.42079641



(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 63X used
(4) network fixed at about 6 minutes (formed, but moved until stationary at 6min)
(5) images taken for 25min due to inconsistencies between images at times.
(6) plateau confirmed by 25min

Trial 4

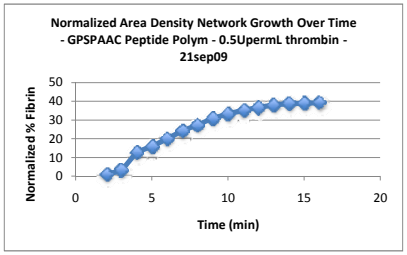
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	0.2439	1
2	10.5074	43.0807708
3	20.7751	85.1787618
4	31.925	130.893809
5	44.4412	182.210742
6	54.7674	224.548585
7	61.2693	251.206642
8	66.7806	273.803198
9	67.1369	275.264043
10	67.9058	278.416564
11	71.4488	292.943009
12	85.3491	349.934809
13	82.2894	337.389914
14	70.3311	288.360394
15	67.3972	276.331283
16	72.0148	295.263633



(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 63X used
(4) very thin and hairy strands - concentrations seem to have been off as compared to usual density of fibers

Trial 5

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	4.2025	1.96222627
2	2.1417	1
3	6.6856	3.12163235
4	27.2847	12.7397395
5	33.8873	15.8226175
6	42.9276	20.0437036
7	51.7847	24.1792501
8	58.8617	27.4836345
9	66.193	30.9067563
10	71.2805	33.2822057
11	75.2728	35.1462857
12	78.7846	36.7860111
13	81.4113	38.0124667
14	83.1844	38.8403605
15	83.4605	38.9692767
16	83.9935	39.2181445

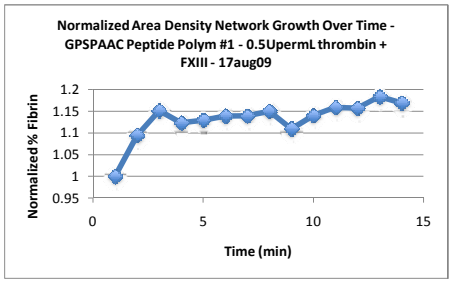


(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) gel network formation @ 2min
(4) very dense network looked smeared
(5) polymerization nearly linear hitting a plateau at a seemingly large % area due to smears of complete fibers

GPSPAAC peptide polymerization
0.5 UpermL thrombin + FXIII

Trial 1

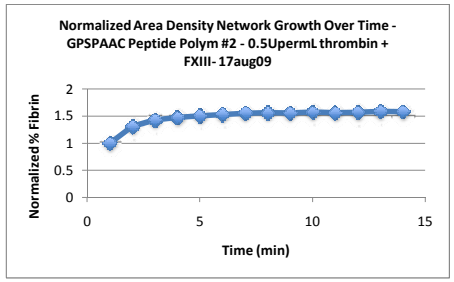
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	57.6527	1
2	63.124	1.09490102
3	66.3985	1.15169801
4	64.7552	1.12319458
5	65.1154	1.12944233
6	65.6741	1.13913312
7	65.6913	1.13943146
8	66.3066	1.15010398
9	63.9677	1.1095352
10	65.7403	1.14028137
11	66.8293	1.15917034
12	66.7433	1.15767865
13	68.2417	1.18366876
14	67.4085	1.16921671



(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 100X used
(4) fibers more webbish (interlaced more evenly)
(5) some clotting that dispersed into a network interlacing over time

Trial 2

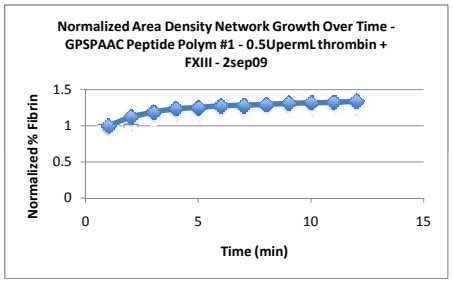
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	39.5125	1
2	51.7691	1.31019551
3	56.4042	1.42750269
4	58.2708	1.47474344
5	59.5867	1.50804682
6	60.5084	1.53137362
7	61.3826	1.55349826
8	61.7923	1.56386713
9	61.5398	1.55747675
10	62.2688	1.57592661
11	61.7455	1.5626827
12	62.0693	1.57087757
13	62.7916	1.58915786
14	62.5098	1.58202594



(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 63X used
(4) formation more easily observed over time as compared to 100X
(5) hairy fibers at first that combined into swoopy networks over time
(6) some clotting apparent at first, but dispersed later

Trial 3

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	64.965	1
2	73.1429	1.12588163
3	77.3312	1.19035173
4	80.5391	1.23973062
5	81.2365	1.25046564
6	83.096	1.27908874
7	83.2202	1.28100054
8	84.2193	1.29637959
9	85.1885	1.31129839
10	85.7621	1.32012776
11	86.053	1.32460556
12	87.0849	1.34048949

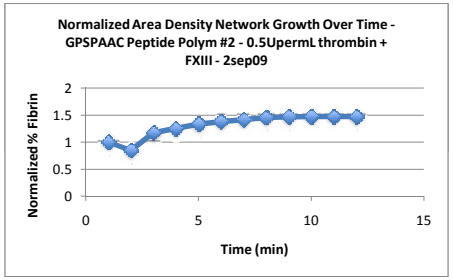


y=63(1.25)^x

(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 100X used
(4) fibers more webbish (interlaced more evenly)
(5) some clotting that dispersed into a network interlacing over time

Trial 4

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	63.5005	1
2	53.8551	0.84810513
3	74.4483	1.17240494
4	79.6638	1.25453815
5	84.6069	1.33238163
6	87.8297	1.38313399
7	90.0863	1.41867072
8	92.4985	1.45665782
9	93.3142	1.46950339
10	93.7427	1.47625137
11	93.8379	1.47775057
12	93.5241	1.47280888

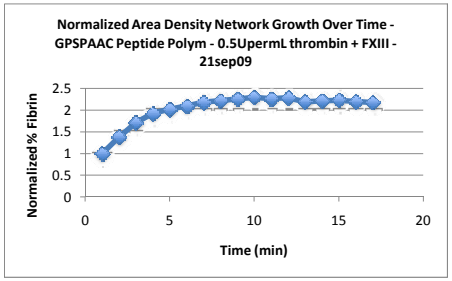


y=62(1.34)^x

(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) 100X used
(4) fibers more webbish (interlaced more evenly)
(5) some clotting that dispersed into a network interlacing over time

Trial 5

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	32.0135	1
2	44.2184	1.38124229
3	54.8423	1.71309916
4	61.6471	1.92565949
5	64.6722	2.020154
6	67.0365	2.09400722
7	69.9098	2.18375998
8	71.0605	2.21970419
9	72.2947	2.25825667
10	73.5773	2.29832102
11	72.4856	2.26421978
12	73.2092	2.28682275
13	70.3035	2.19605791
14	70.9049	2.21484374
15	71.3861	2.2298749
16	70.3865	2.19865057
17	69.7451	2.17861527



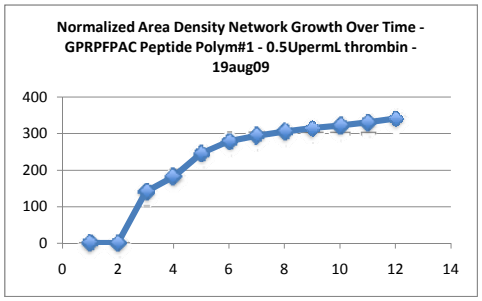
y=62(1.34)^x

(1) prep standard
(2) pep. 1 gel - controlled Ca
(3) gel network formation @ 2min
(4) very short strands arranged in clusters - cluttered

GPRPFPAC peptide polymerization
0.5 UpermL thrombin

Trial 1

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	0.1351	1
2	0.2195	1.62472243
3	19.0147	140.745374
4	24.6125	182.179867
5	33.2114	245.828275
6	37.7946	279.752776
7	39.753	294.248705
8	41.3167	305.823094
9	42.5546	314.985936
10	43.548	322.339008
11	44.6602	330.571429
12	46.1286	341.440415

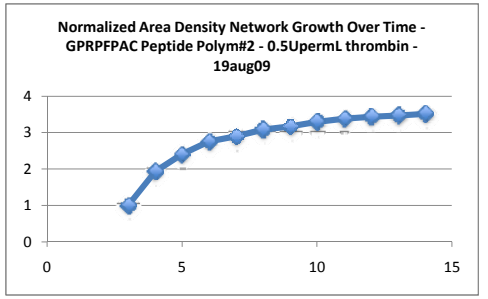


(1) prep standard - good flow since holes were re-bore to clear up any re-curing of devices
(2) pep.3 gel - controlled Ca
(3) some clotting around needle once thrombin containing needle inserted into pep. 3 gel
(4) first observed growth @ around 3 min
(5) growth continued and observed till 14 minutes when movement up z-stack was required
(6) additional images taken @ 2 min intervals to ensure growth completed.
(7) pinhole = 0.8

0+(50/(1+((Time/6)^-0.5)))

Trial 2

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	48.6107	3.12083178
2	66.8205	4.28991025
3	15.5762	1
4	30.0279	1.92780653
5	37.4939	2.40712754
6	42.9688	2.75861892
7	45.1406	2.89804959
8	48.0825	3.08692107
9	49.4123	3.17229491
10	51.3067	3.29391636
11	52.6841	3.38234614
12	53.4669	3.4326023
13	53.9721	3.4650364
14	54.6879	3.51099113

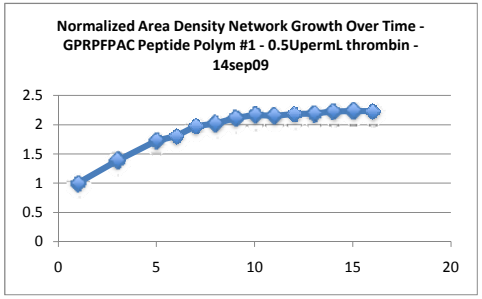


(1) prep standard - holes re-bore
(2) pep.3 gel - controlled Ca
(3) images taken every minute for 30 min, followed by 35 & 40 min images
(4) first observed growth @ around 3 min
(5) steady growth rate hit plateau
(6) no movement in z direction necessary
(7) pinhole = 1.7

0+(55/(1+((Time/7)^-0.5)))

Trial 3

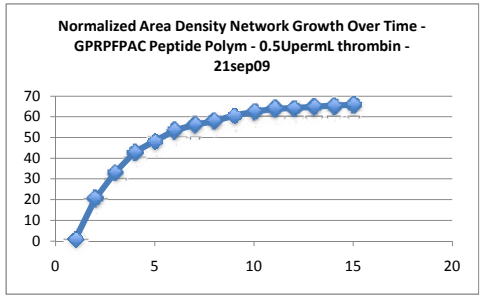
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	21.6021	1
3	29.9678	1.38726328
5	37.2178	1.72287879
6	38.9878	1.80481527
7	42.727	1.97790956
8	43.6227	2.01937312
9	45.6071	2.11123456
10	46.8612	2.1692891
11	46.4956	2.15236482
12	47.2008	2.18500979
13	47.2612	2.18780582
14	48.1048	2.22685757
15	48.2806	2.23499567
16	48.2098	2.23171821



(1) prep standard - good flow since holes were re-bore to clear up any re-curing of devices
(2) pep.3 gel - controlled Ca
(3) large scale clotting of peptide w/ fibrinogen once thrombin containing needle inserted into pep. 3 gel
(4) channel reinjected 30 min after 1st inject since clotting was observed b/w 1st fibrinogen and peptide sol
(5) observed growth @ 1min once channel re-injected
(6) images taken for 17min ensuring complete growth
(7) pinhole = 1.7

Trial 4

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	0.8672	1
2	17.7719	20.4934271
3	28.8031	33.2139068
4	37.3153	43.0296356
5	41.8478	48.2562269
6	46.3085	53.4000231
7	48.6569	56.1080489
8	50.2289	57.9207795
9	52.5726	60.6233856
10	54.0289	62.3026983
11	55.3853	63.8668127
12	55.7379	64.2734087
13	56.1604	64.7606089
14	56.7009	65.3838792
15	56.9896	65.7167897



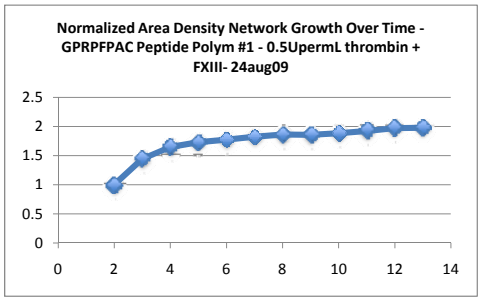
(1) prep standard - holes re-bore
(2) pep.3 gel - controlled Ca
(3) images taken every minute for 15 min
(4) first observed growth @ around 2 min
(5) steady growth rate hit plateau
(6) no movement in z direction necessary
(7) pinhole = 1.7
(8) many clots observed at 2 mins dispersed into a network over time - not fully but partially

0+(55/(1+((Time/7)^-0.5)))

GPRPFPAC peptide polymerization
0.5 UpermL thrombin + FXIII

Trial 1

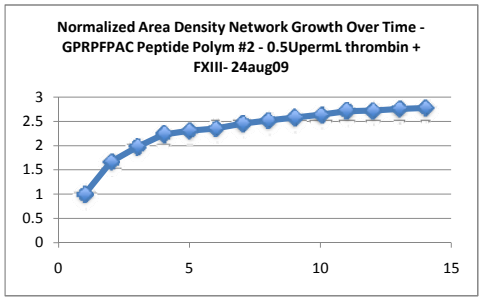
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	31.8786	1
3	46.0851	1.44564379
4	52.4954	1.64672853
5	55.1506	1.73001951
6	56.6516	1.77710439
7	58.176	1.8249233
8	59.3246	1.86095374
9	59.2283	1.85793291
10	60.1278	1.88614933
11	61.3383	1.92412151
12	62.9553	1.97484519
13	63.1059	1.97956937



(1) prep standard - leak observed once rinsed with TBS + CaCl2, but still imaged b/c leak was contained by pressing onto the device gently to seal the edges
(2) pep. 3 gel - controlled Ca
(3) gel sat for 2.5 hours prior to imaging
(4) gel formation after 2 minutes
(5) hairy like
(6) dense in some areas
(7) resembles fibrin network w/o peptide very closely (fibers do not look coated)
(8) threshold - 15.0

Trial 2

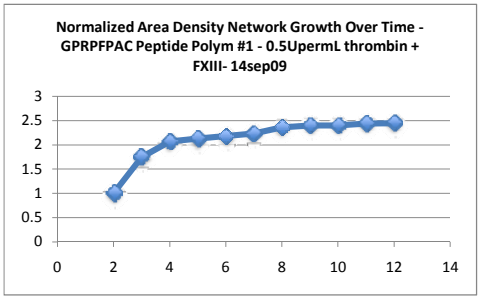
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	25.3998	1
2	42.327	1.66643044
3	50.1193	1.97321632
4	56.6404	2.22995457
5	58.5471	2.30502209
6	59.8215	2.35519571
7	62.2758	2.45182246
8	63.9725	2.51862219
9	65.5299	2.57993764
10	67.0304	2.63901291
11	68.823	2.70958826
12	69.156	2.7226986
13	69.9832	2.75526579
14	70.4641	2.77419901



(1) prep standard - no leaks
(2) pep. 3 gel - controlled Ca
(3) gel clumped, so remade and sat for 30 minutes
(4) gel formation after 2 minutes
(5) not as hairy like as prior set of images
(6) short fibers with random interlacing
(7) not very dense
(8) threshold - 12.0

Trial 3

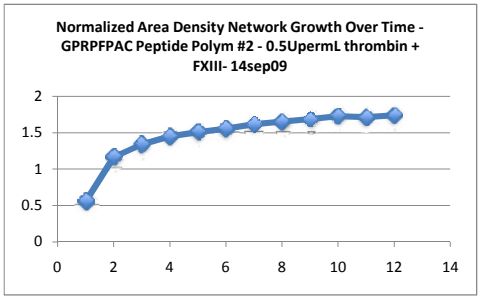
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	26.6293	1
3	46.494	1.74597154
4	55.0746	2.06819556
5	56.6185	2.12617305
6	58.054	2.18007984
7	59.5554	2.23646134
8	62.903	2.36217249
9	63.9274	2.4006414
10	63.934	2.40088925
11	65.0132	2.44141603
12	65.1681	2.44723294



(1) prep standard - no leaks
(2) pep. 3 gel - controlled Ca
(3) clots formed in a few areas and grew slightly over time
(4) gel formation after ~3 minutes
(5) fibers almost all project from these few clot points
(6) fibers progress from thick to thin moving out from the initial location of formation
(7) dense network interlacing with thick joint points

Trial 4

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	17.8083	0.55862867
2	37.1459	1.16522997
3	42.7263	1.34028157
4	46.2354	1.45035855
5	48.0705	1.50792381
6	49.5605	1.55466363
7	51.6058	1.61882266
8	52.6312	1.65098844
9	53.8682	1.6897919
10	55.045	1.72670694
11	54.6483	1.71426286
12	55.4742	1.74017052

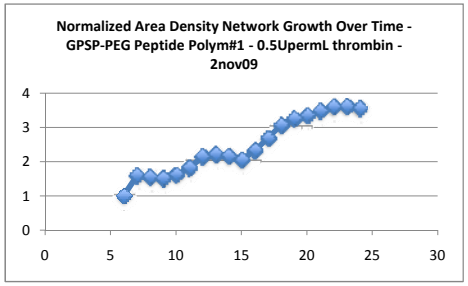


(1) prep standard - no leaks
(2) pep. 3 gel - controlled Ca
(3) gel clumped to a large scale at the bottom right
(4) gel formation after 2 minutes
(5) fibers thick at joints and relatively thin when far from a clots/joints

GPSP-PEG peptide polymerization
0.5 UpermL thrombin

Trial 1

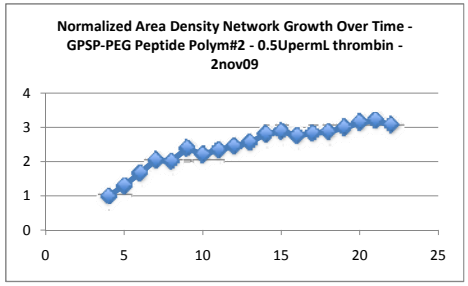
Time (min)	MATLAB percent fibrin	normalized percent fibrin
6	17.1462	1
7	27.028	1.57632595
8	26.3521	1.53690614
9	25.5894	1.49242398
10	27.3914	1.59752015
11	31.0143	1.80881478
12	36.6174	2.13559856
13	37.6486	2.19574016
14	36.7788	2.14501172
15	34.9115	2.03610713
16	39.6585	2.31296147
17	45.8868	2.67620814
18	52.237	3.04656425
19	55.5605	3.24039729
20	57.2088	3.33652938
21	59.5368	3.4723029
22	61.4799	3.5856283
23	61.7444	3.60105446
24	60.7864	3.54518202



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7

Trial 2

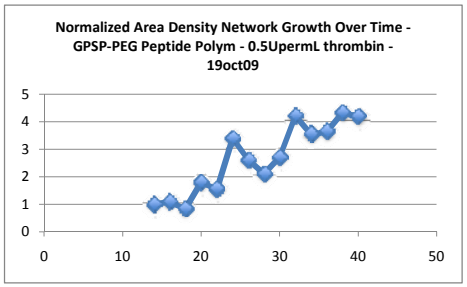
Time (min)	MATLAB percent fibrin	normalized percent fibrin
4	16.5284	1
5	21.596	1.30659955
6	27.6225	1.67121439
7	33.742	2.04145592
8	33.2195	2.00984366
9	39.5101	2.39043707
10	36.5071	2.20874979
11	38.6216	2.33668111
12	40.5027	2.45049128
13	42.2294	2.55495995
14	46.2605	2.79884925
15	47.5913	2.87936521
16	45.5969	2.75870018
17	46.685	2.82453232
18	47.4505	2.87084654
19	49.3295	2.98452966
20	51.8604	3.13765398
21	52.9018	3.20066068
22	50.5894	3.06075603



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 16 minutes
(3) pinhole = 1.7

Trial 3

Time (min)	MATLAB percent fibrin	normalized percent fibrin
14	6.5446	1
16	7.1158	1.08727806
18	5.4399	0.83120435
20	11.693	1.78666381
22	10.1226	1.54671026
24	22.1991	3.39197201
26	17.0583	2.60646946
28	13.7937	2.107646
30	17.7126	2.70644501
32	27.5343	4.20717844
34	23.1531	3.53774104
36	23.7772	3.6331021
38	28.3068	4.32521468
40	27.362	4.18085139

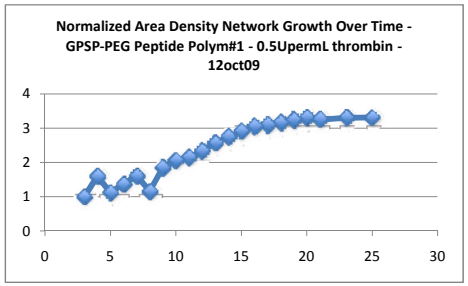


(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7
(4) not consistent w/ resolution but growth over time apparent-try to fit a logistic to it

GPSP-PEG peptide polymerization
0.5 UpermL thrombin + FXIII

Trial 1

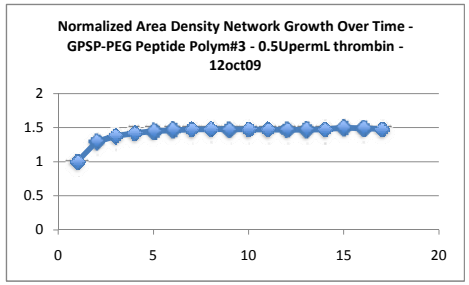
Time (min)	MATLAB percent fibrin	normalized percent fibrin
3	15.8464	1
4	25.2157	1.59125732
5	17.8047	1.12358012
6	21.6473	1.36607053
7	25.5049	1.60950752
8	18.3729	1.15943684
9	29.3064	1.84940428
10	32.642	2.05990004
11	34.0458	2.14848798
12	36.9505	2.33179145
13	40.6279	2.56385678
14	43.4257	2.74041423
15	45.8668	2.89446183
16	48.0947	3.03505528
17	48.9325	3.08792533
18	49.9203	3.15026126
19	51.0513	3.22163394
20	52.2709	3.29859779
21	51.4525	3.24695199
23	52.2403	3.29666675
25	52.3945	3.30639767



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7
(4) okay-shaky at first but fine after

Trial 2

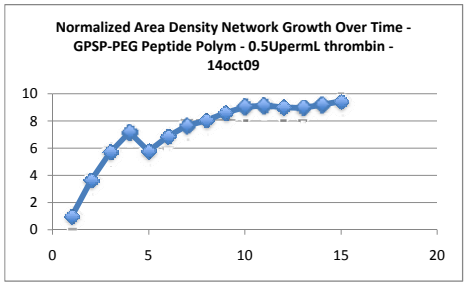
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	34.4265	1
2	44.441	1.2908951
3	47.4227	1.3775057
4	48.9469	1.42177973
5	49.8347	1.44756801
6	50.287	1.46070614
7	50.6641	1.47165991
8	51.0551	1.48301744
9	50.7348	1.47371356
10	50.8312	1.47651373
11	50.7241	1.47340276
12	50.7057	1.47286828
13	50.4551	1.46558901
14	50.8376	1.47669964
15	51.6037	1.49895284
16	51.2556	1.48884144
17	50.8985	1.47846862



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7
(4) good-easy to model (although not very much growth past initial mins)

Trial 3

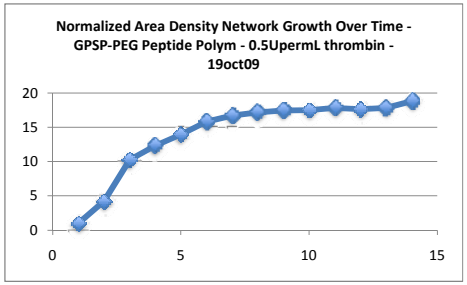
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	3.6243	1
2	13.1995	3.64194465
3	20.7389	5.72218083
4	25.9904	7.17115029
5	20.9692	5.78572414
6	24.9125	6.87374114
7	27.7432	7.65477472
8	29.1618	8.04618823
9	31.172	8.60083326
10	32.7351	9.03211655
11	33.1125	9.136247
12	32.6385	9.00546312
13	32.4526	8.95417046
14	33.2877	9.18458737
15	34.2164	9.44082995



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 19 minutes
(3) pinhole = 1.7
(4) image at 4min off

Trial 4

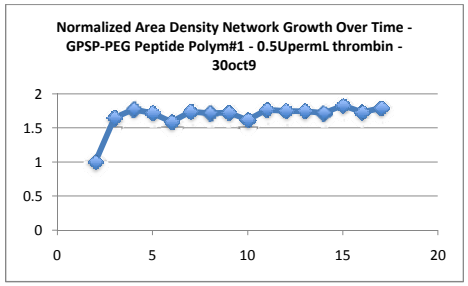
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	2.8572	1
2	12.0872	4.23043539
3	29.3355	10.2672197
4	35.1644	12.3072939
5	39.8759	13.9562859
6	45.1859	15.8147487
7	47.7987	16.7292104
8	49.0905	17.1813314
9	49.9256	17.4736105
10	50.0878	17.5303794
11	51.0104	17.8532829
12	50.4677	17.6633417
13	50.9254	17.8235335
14	53.7201	18.801659



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7

Trial 5

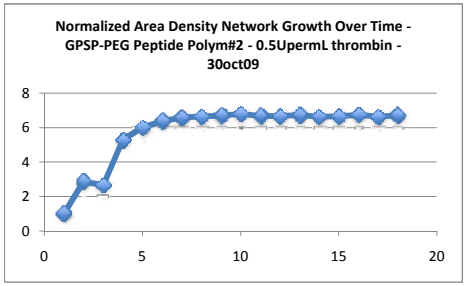
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	37.0339	1
3	61.0018	1.64718812
4	65.5575	1.77020244
5	63.5411	1.71575502
6	58.7717	1.58697032
7	64.3925	1.73874477
8	63.5625	1.71633287
9	63.7885	1.72243539
10	59.8676	1.61656212
11	65.3282	1.76401081
12	64.7463	1.74829818
13	64.6084	1.74457457
14	63.5947	1.71720235
15	67.5828	1.82489017
16	63.9731	1.72742001
17	66.1742	1.78685475



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 16 minutes
(3) pinhole = 1.7
(4) good looking images

Trial 6

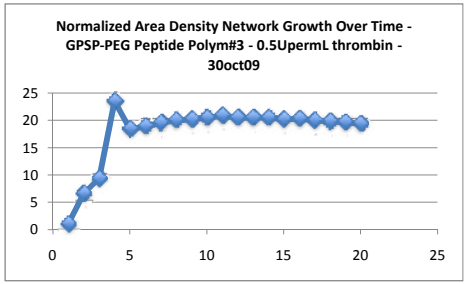
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	9.161	1
2	26.1697	2.85664229
3	24.3431	2.65725357
4	48.4766	5.29162755
5	55.0018	6.00390787
6	58.3816	6.37284139
7	60.3149	6.58387731
8	60.8725	6.64474402
9	61.277	6.68889859
10	62.313	6.80198668
11	61.0882	6.66828949
12	61.0652	6.66577885
13	61.6179	6.72611069
14	60.8098	6.63789979
15	60.9094	6.64877197
16	61.8295	6.7492086
17	60.7507	6.63144853
18	61.4792	6.71097042



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 16 minutes
(3) pinhole = 1.7
(4) good looking images

Trial 7

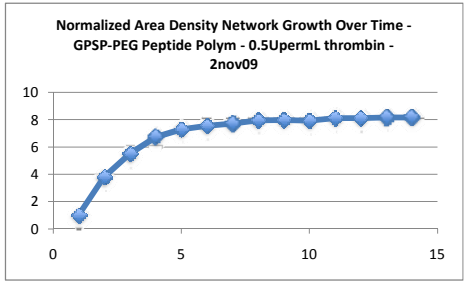
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	2.1333	1
2	14.1294	6.62325974
3	19.9878	9.36942765
4	50.1287	23.4981953
5	39.2781	18.4118971
6	40.2907	18.8865607
7	41.6896	19.5423053
8	42.7012	20.0165003
9	43.0015	20.1572681
10	43.6727	20.471898
11	44.5086	20.8637322
12	43.7033	20.486242
13	43.6468	20.4597572
14	43.7218	20.494914
15	43.0439	20.1771434
16	43.3131	20.3033329
17	42.6124	19.9748746
18	42.191	19.7773403
19	41.874	19.6287442
20	41.3148	19.3666151



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 16 minutes
(3) pinhole = 1.7
(4) good looking images

Trial 8

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	8.7945	1
2	33.5541	3.8153505
3	48.4358	5.50751038
4	59.1399	6.72464609
5	64.1809	7.29784524
6	66.4499	7.5558474
7	67.8216	7.71181989
8	70.0092	7.96056626
9	70.1567	7.97733811
10	69.7563	7.93180965
11	71.3827	8.11674342
12	71.3355	8.11137643
13	71.7162	8.15466485
14	71.7768	8.16155552

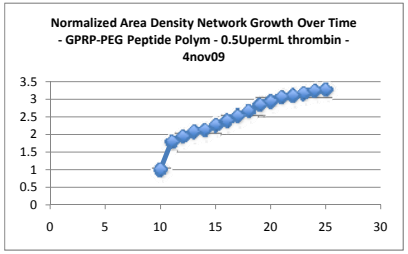


(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 16 minutes
(3) pinhole = 1.7

GPRP-PEG peptide polymerization
0.5 UpermL thrombin

Trial 1

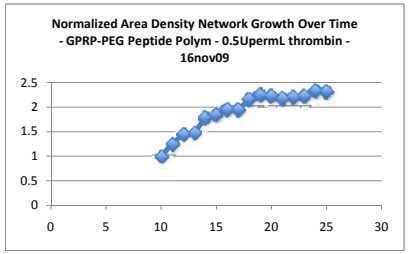
Time (min)	MATLAB percent fibrin	normalized percent fibrin
10	13.1068	1
11	23.4355	1.78804132
12	25.4706	1.94331187
13	27.2831	2.08159886
14	27.9155	2.12984863
15	29.785	2.27248451
16	31.3694	2.39336833
17	33.1463	2.52893918
18	35.0349	2.67303232
19	37.3138	2.8469039
20	38.5569	2.9417478
21	40.1909	3.06641591
22	40.7617	3.10996582
23	41.6285	3.17609943
24	42.6019	3.25036622
25	43.0164	3.28199103



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7

Trial 2

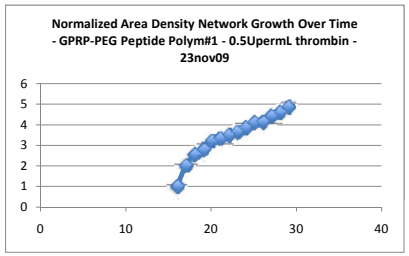
Time (min)	MATLAB percent fibrin	normalized percent fibrin
10	20.049	1
11	25.0987	1.25186792
12	29.0691	1.44990274
13	29.644	1.47857749
14	35.6932	1.78029827
15	36.9225	1.84161305
16	39.0728	1.94886528
17	39.0259	1.94652601
18	43.2352	2.15647663
19	45.0814	2.24856103
20	44.5192	2.22051973
21	43.5222	2.17079156
22	44.1404	2.20162602
23	44.602	2.22464961
24	46.6319	2.32589655
25	46.3773	2.31319767



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.2

Trial 3

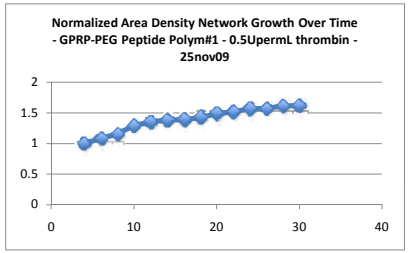
Time (min)	MATLAB percent fibrin	normalized percent fibrin
16	8.3049	1
17	16.9295	2.03849535
18	21.3279	2.56811039
19	23.3495	2.81153295
20	26.8461	3.2325615
21	27.847	3.35308071
22	29.2263	3.51916339
23	30.4806	3.6701947
24	32.1449	3.87059447
25	34.2294	4.12159087
26	34.5106	4.1554504
27	36.9558	4.44987899
28	38.3542	4.61826151
29	40.5487	4.8825031



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 16 minutes
(3) pinhole = 1 AU

Trial 4

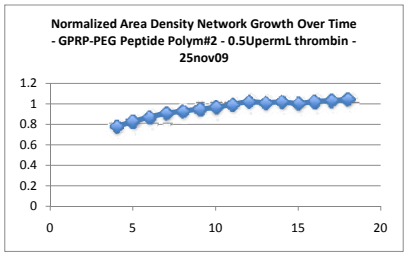
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	69.8438	1.86107203
4	37.5288	1
6	40.6867	1.08414604
8	43.3656	1.15552855
10	48.5609	1.29396357
12	50.6101	1.34856697
14	51.9979	1.38554657
16	52.0703	1.38747575
18	53.4509	1.4242635
20	55.7311	1.48502217
22	56.8744	1.51548677
24	58.6509	1.56282375
26	59.0535	1.57355151
28	60.4896	1.61181812
30	60.9802	1.62489075



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) polymerization started at about 4 minutes
(3) pinhole = 1 AU

Trial 5

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	59.2748	1
2	52.5742	0.88695702
3	48.3784	0.81617146
4	46.2861	0.78087315
5	48.9981	0.82662615
6	51.7965	0.87383677
7	53.7767	0.90724389
8	55.1365	0.9301845
9	56.206	0.94822758
10	57.1982	0.96496656
11	58.9644	0.99476337
12	60.7592	1.02504268
13	59.8665	1.00998232
14	60.4896	1.02049438
15	59.4192	1.00243611
16	60.675	1.02362218
17	61.1629	1.03185333
18	61.8782	1.04392086

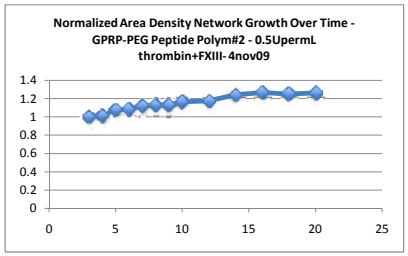


(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) polymerization started at about 4 minutes
(3) pinhole = 1 AU

GPRP-PEG peptide polymerization
0.5 UpermL thrombin + FXIII

Trial 1

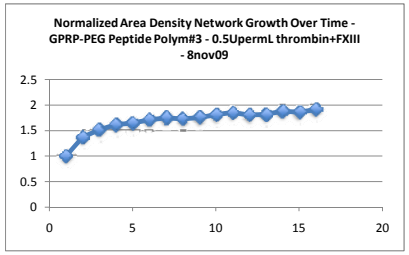
Time (min)	MATLAB percent fibrin	normalized percent fibrin
2	68.7141	1.17109273
3	58.6752	1
4	59.4669	1.01349292
5	63.2443	1.07787106
6	63.744	1.08638743
7	65.6557	1.11896849
8	66.3182	1.13025946
9	66.2964	1.12988793
10	68.4181	1.16604801
12	69.0545	1.17689416
14	72.8809	1.2421074
16	74.3725	1.2675287
18	73.3771	1.25056412
20	74.0423	1.26190111



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) no polymerization till about 11 minutes
(3) pinhole = 1.7

Trial 2

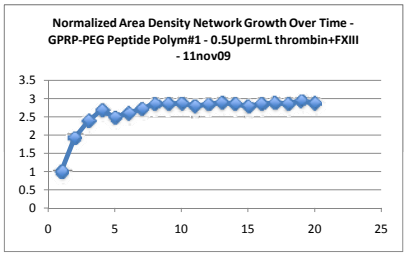
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	31.3301	1
2	43.0631	1.37449609
3	47.4666	1.51504783
4	50.5436	1.61326009
5	51.5401	1.64506657
6	53.6337	1.71189048
7	54.8056	1.74929541
8	54.2514	1.73160635
9	54.9835	1.75497365
10	56.6409	1.80787486
11	58.086	1.85399983
12	56.59	1.80625022
13	56.859	1.81483621
14	58.824	1.87755545
15	58.3109	1.86117823
16	59.9066	1.91211008



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) polymerization started at about 4 minutes
(3) pinhole = 1.7

Trial 3

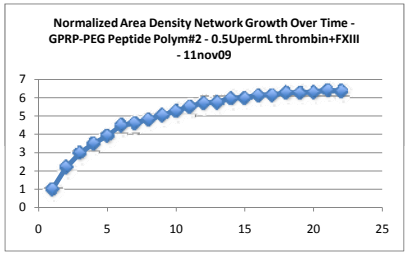
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	20.3814	1
2	39.3657	1.93145221
3	48.6945	2.38916365
4	54.6762	2.68265183
5	50.6867	2.48690963
6	52.9214	2.59655372
7	55.4376	2.72000942
8	58.1048	2.85087384
9	58.1461	2.85290019
10	58.5475	2.87259462
11	57.0238	2.79783528
12	57.9249	2.84204716
13	58.9305	2.89138626
14	58.1524	2.8532093
15	56.8472	2.78917052
16	58.1515	2.85316514
17	58.7676	2.88339368
18	58.2579	2.85838559
19	60.0448	2.94605866
20	58.5839	2.87438056



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) polymerization started at about 4 minutes
(3) pinhole = 1.2

Trial 4

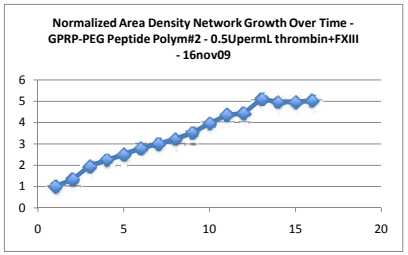
Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	11.5809	1
2	25.4402	2.19673773
3	34.4155	2.97174658
4	40.5356	3.50021156
5	45.392	3.9195572
6	52.1221	4.50069511
7	53.4364	4.6141837
8	55.8456	4.82221589
9	58.4095	5.04360628
10	61.2063	5.28510738
11	63.9987	5.52622853
12	66.2131	5.71743992
13	66.5769	5.74885372
14	69.2422	5.97899991
15	69.4674	5.99844572
16	71.3543	6.16137779
17	71.4072	6.16594565
18	72.8052	6.28666166
19	72.738	6.280859
20	73.0981	6.3119533
21	74.4142	6.42559732
22	73.8459	6.37652514



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) polymerization started at about 4 minutes
(3) pinhole = 1.2

Trial 5

Time (min)	MATLAB percent fibrin	normalized percent fibrin
1	6.8459	1
2	9.0476	1.32160855
3	13.4522	1.96500095
4	15.3103	2.23641888
5	17.1097	2.49926233
6	19.2139	2.80662879
7	20.4852	2.99233118
8	22.0761	3.22471844
9	24.1286	3.52453293
10	27.0945	3.95777034
11	29.8022	4.35329175
12	30.3862	4.43859829
13	34.9425	5.10414993
14	33.9622	4.96095473
15	33.9398	4.9576827
16	34.3776	5.02163339



(1) large amount of background noise under normal threshold - adjusted to >=25 in matlab
(2) polymerization started at about 4 minutes
(3) pinhole = 1.7
(4) very light/hazy growth