Visualization and identification of the structures formed during early stages of fibrin polymerization

**Running title:** IMAGING EARLY STAGES OF FIBRIN POLYMERIZATION

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Abstract

We determined the sequence of events, identified structures and quantitatively characterized the mobility of moving structures present during early stages of fibrin clot formation from the beginning of polymerization to the gel point, using three complementary techniques in parallel, spinning disk confocal microscopy, transmission electron microscopy and turbidity measurements. At the beginning of polymerization, the major structures were monomers, while at the middle of the lag period there were monomers, oligomers, protofibrils and fibers. At the end of the lag period, there were primarily monomers and fibers, giving way to mainly fibers at the gel point. Diffusion rates were calculated from two different results, one based on sizes and another from velocity of observed structures, with similar results in the range of 3.8 - 0.1 µm²/sec. At the gel point, the diffusion coefficients corresponded to very large, slow moving structures, as well as individual protofibrils. The smallest moving structures visible by confocal microscopy during fibrin polymerization were identified as protofibrils with a length of about 0.5 µm. The sequence of early events of clotting and the structures present determined from these studies are important for understanding hemostasis and thrombosis.
Introduction

Fibrin clot formation is a stepwise process, the first step being cleavage of fibrinogen by thrombin to remove fibrinopeptides A (FpA), which exposes the knobs ‘A’ binding sites to form fibrin monomers\textsuperscript{1-3}. The fibrin monomers polymerize in a half – staggered manner producing small oligomers and protofibrils\textsuperscript{4-7} with a periodicity of 22.5 nm, as shown by using electron microscopy and X-ray diffraction\textsuperscript{8}. The next step is the association of protofibrils side by side, or lateral aggregation. To proceed from longitudinal to lateral growth, protofibrils must be sufficiently long, approximately in the range of 0.6 $\mu$m to 0.8 $\mu$m\textsuperscript{9}. Then, the fibrin network is established through fiber branching, lateral and longitudinal growth, resulting in a gel\textsuperscript{10}. Finally, there is some network rearrangement, due to new fiber formation\textsuperscript{11}. The rate of polymerization is affected by many factors, including the rate of fibrinopeptide cleavage, oligomer formation and lateral aggregation\textsuperscript{12}. Recently, we described the sequence of events during fibrin network formation in the hydrated clot, observed by deconvolution microscopy in the real time of polymerization\textsuperscript{11}.

Fibrin polymerization is a dynamic process, which makes it difficult to study. We found that at the early stages of network formation, fibers are mobile\textsuperscript{11}, but we were not able to characterize the movements of the fibers quantitatively. To quantify this type of movement, it is necessary to collect a large stack of images in a short time. To resolve this issue, in the research reported here we used a spinning disk confocal microscope, which has the advantage compared to the deconvolution microscope that it has multiple pinholes in a disk rotating as rapidly as 1800 to 5000 rpm, providing very fast, video rate image acquisition.

However, the resolution limit of any light microscope does not allow the detailed visualization of small structures, such as fibrin monomers, dimers, trimers and tetramers, which appear at the beginning of polymerization. To visualize such small structures that formed at different times until the gel point, transmission electron microscopy has been used. Thus, in the present work spinning disk confocal
microscopy, electron microscopy and turbidity measurements have been correlated to monitor fibrin polymerization dynamically with good temporal and spatial resolution. The sequence of early events of fibrin clot formation and changes in proportion of structures, monomers, dimers, trimers, protofibrils and fibers, were characterized as a function of time from the beginning of polymerization to the gel point. The mobility and sizes of fibrin-related structures were characterized quantitatively, and the smallest moving structures visible in the light microscope were identified. The new findings bring us closer to understanding the early stages of clot formation, which may help to interpret the clinical significance of changes in the gel point and lead to new methods of treatment and prophylaxis of thrombosis.
Materials and Methods

Fibrin Polymerization Reaction and Gel Point Measurements

Polymerization was carried out in 50 mM Tris-HCl, 140 mM NaCl, pH 7.4, 2.5 mM CaCl₂ by the mixing of 1.5 mg/ml purified human plasminogen-free fibrinogen from Hyphen BioMed (France) and 0.1 U/ml thrombin (final concentrations). Alexa 488 labeled fibrinogen (Invitrogen, Carlsbad, CA 92009) with an average of 6 molecules of dye per molecule of protein was added to the polymerization mixture at a final concentration of 0.03 mg/ml. After all components were mixed, the sample was inserted into a Sigmacoat-treated glass chamber made of a microscope slide and a cover slip separated by two strips of double-stick tape, such that the height of the chamber was 350 μm. Sigmacoat was used to produce a very thin hydrophobic film on the glass surface to reduce surface effects.

The time of the change from the liquid to the solid state is called the gel point. To measure the gel point, the polymerization mixture as described above was prepared in transparent Sigmacoat-treated glass tubes. A timer was started after all components were mixed, and the gel point was observed by eye, with 5 replicates for each sample.

Light Microscopy Studies

Polymerization reactions were carried out as described above in the Fibrin Polymerization Reaction and Gel Point Measurements section. A stack of 26 images was acquired in real time during the fibrin polymerization reaction, every 16 seconds for 45 minutes by using a spinning disk confocal microscope consisting of a Yokogawa CSU 10 scanner combined with an Olympus IX 71 microscope using a 63 X 1.4 NA Plan Apo or 20X objective lens (Olympus America, Inc., Center Valley, PA). A Hammamatsu Image back – thinned EMCCD camera (C9100 – 13) was used for image capture (Hamamatsu,
Quantitative Analysis and Identification of Moving Structures

Diffusion rates have been calculated to quantitatively characterize moving structures. Three different approaches were used to calculate diffusion rates.

One approach was based on the velocity of the moving structures observed. The distance that represents the displacement of the same structure over a given time was measured from two different images obtained with a known time interval and the velocity was calculated.

The second approach was based on measurement of the size of structures observed. The size measurement of the structures was obtained from spinning disk confocal micrographs and corrected by using the point spread function, or the degree of spreading (blurring) of the point object. The point spread function was measured with 210 nm beads as point objects, using methods that have been developed and validated by others to correct the size measurements of fluorescent structures observed by light microscopy\textsuperscript{13,14,15,16}. Z stacks of bead images were merged into one and the intensity profile was built, using a Gaussian function to fit the intensity profile. To determine the apparent size of the bead, the circle within which the intensity is 50\% of the maximum intensity, the full width at half maximum, was measured. ImageJ 1.36 b (NIH, Bethesda, USA) software was used for analysis of the light micrographs.

The third approach was based on comparisons of diffusion coefficients obtained from experimental data with calculated diffusion coefficients of various size protofibrils with different diameters and lengths. The average diameter of a cylindrical protofibril was calculated, assuming that the average density of protein is 0.73 g/cm\textsuperscript{3} and knowing the molecular weight of fibrin to calculate the volume, with two halves of a fibrin molecule within each 2.25 nm repeat. Then, knowing the diameter of an individual protofibril and taking into account the information that lateral aggregation starts after the
protofibril reaches a length between 600 nm – 800 nm, we calculated the diameters and lengths for two, three, four and five protofibrils aggregated laterally. The calculations for oligomers and protofibrils are straightforward because we know the sizes and protein content of these structures accurately. The best model that we have of initial lateral aggregates is that the protofibrils twist around each other, so we have calculated an average diameter assuming that they are roughly cylindrical. The diameters were corrected for the measured relative amount of protein and water in fibrin fibers. We used these sizes to calculate the diffusion rates that could be employed like a ruler to define the types of structures compared to diffusion rates based on experimental data.

Transmission Electron Microscopy Studies

The polymerization reaction was carried out as described in the Polymerization Reaction and Gel Point Measurements section and was stopped at the specific times, 0, 1, 3, 6 and 9 min by addition of 5 units/ml final concentration of hirudin (Sigma – Aldrich, St. Louis, MO 63178, US) and immediately diluted with a volatile buffer, 0.05 M ammonium formate with 30% glycerol. Very rapid dilution and spraying of the samples minimized the potential for dissociation of oligomers, but some changes may still occur. Samples were rotary-shadowed after spraying diluted samples onto freshly cleaved mica by shadowing with tungsten in a vacuum evaporator (Denton Vacuum, Cherry Hill, NJ). Specimens were examined in an FEI/Philips 400 electron microscope (FEI, Hillsboro, OR). Images were taken at 80 kV with a magnification of 36 000 x. More than 10 micrographs were analyzed at each time point.

Analysis of Electron Micrographs

The amount of monomers, dimers, trimers, tetramers, protofibrils, and fibers were determined from counting of structures observed in transmission electron microscope images of the rotary shadowed structures described above. A protofibril was defined as a double strand of 8 or more monomers. These measurements were carried out for at least 10 electron micrographs for each time point (1, 3, 6 and 9
minutes). Once the structures present were counted, all counts were normalized by the total amount of monomers present in all of the structures.

**Results**

*Turbidity Measurements of Fibrin Polymerization*

The dynamics of fibrin polymerization were characterized in general under the conditions of these microscopy experiments by measurement of turbidity curves, averaged from 3 identical polymerization assays. For direct comparison with the microscopy results, turbidity was measured in the same chambers that were used for microscopy (Fig. 1). The lag period, or the time until the turbidity begins to rise, was determined from the turbidity curves to be 345 sec ± 10 sec. The average gel point, or the time of the change from liquid to solid, was determined to be 540 ± 11 sec (n = 5) (Fig. 1).

*Spinning Disk Confocal Microscopy of Fibrin Polymerization*

Four-dimensional data were obtained from the time of mixing of fibrinogen and thrombin until the gel point and carefully analyzed. We observed fluorescently labeled fibrin structures that were mobile and moved in three dimensions (Supplemental material video 1 and 2). At the beginning of polymerization, the smallest structures appeared as small fluorescent dots moving among larger soluble fibrin precursors. At the middle of the lag period at approximately 3 min, these structures elongated and appeared as rod-like structures or fibers, which had length and diameter. They continued to move independently of each other and started branching. At the end of the lag period at approximately 5 min, they continued moving but the velocity of these structures slowed down, due to intensive branching and longitudinal and lateral growth. At 6 min, when the turbidity curve started to rise, branching, lateral and longitudinal growth resulted in the formation of a fiber scaffold, which continued to move slowly. At the
time the scaffold was formed, there were some small structures that continued moving with velocities higher than that of the scaffold. They later grew laterally and longitudinally and eventually attached to the scaffold.

**Electron microscopy study of fibrin polymerization**

To answer the question of what were the smallest fibrin-related structures present at the beginning of polymerization and how they changed prior to the gel point, we synchronized our confocal microscopy experiments with electron microscopy experiments. Four time points were chosen, 1, 3, 6, and 9 min, at which the reaction was stopped (Materials and Methods). The 1 minute time point corresponded to the time just after fibrinogen and thrombin were mixed (0 time); 3 minutes corresponded to the middle of the lag phase; 6 minutes corresponded to the end of the lag period when the polymerization curve started to rise, and 9 minutes corresponded to the gel point (Fig. 1).

Structures that appeared at each time point were identified from their morphology as monomers, dimers, trimers, tetramers, or protofibrils (defined as structures that consisted of more than 8 monomers, and fibers. Structures consisting of 5, 6 and 7 monomers were not observed under our experimental conditions (Fig. 2).

To understand the dynamic changes of fibrin structures as a function of time during the early stages of fibrin polymerization, the numbers of each type of observed structure were quantified for each time point. The number of monomers within each type of structure were calculated and normalized by the number of total monomers present in each whole micrograph, determined as the sum of all free monomers plus monomers present in all other fibrin-structures (Fig. 3). The results of such analyses show that at 1 minute from the beginning of polymerization, the major structure was monomers, while small protofibrils consisting of more than 8 fibrin monomers in size represented 2% of all defined structures (Fig. 3). Fibers were not present at this time point. In the middle of the lag period, at 3 min, all types of structures that we described above were found: monomers, dimers, trimers, tetramers,
protofibrils, and fibers. At this time, monomers still remained a major fraction, at 54%. At 6 min, when the turbidity started rising, the amount of free monomers and monomers polymerized in dimers, trimers, tetramers, protofibrils and fibers were almost equal. Around the time of the gel point, at 9 min, the major structures appeared to be fibers, but also some amount of smaller structures were present: monomers, dimers and trimers, although tetramers were not found at this time point.

Quantitative Analysis and Identification of Moving Structures

To characterize the movement and sizes of fibrin structures during the early stages of fibrin polymerization, diffusion coefficients were calculated. Two different approaches have been used to calculate the diffusion rate, one based on size and another one based on the velocity of moving structures. The sizes of moving structures observed in the spinning disc confocal microscope, lengths of rod-like structures, and diameters of round structures, were measured. The measurements from the confocal microscope were corrected using the point spread function of our instrument to compensate somewhat for the imaging limitations of light microscopy (see Materials and Methods section)\textsuperscript{13,19}. We measured the velocity of moving structures during the lag period by identifying the same structures at two close time points (Fig. 4). We observed a decrease of the diffusion rates for a majority of structures from both calculations, based on velocity and based on size, over time until the gel point, probably because of the increase of sizes with time due to the processes of elongation and lateral aggregation (Fig. 5). However, at the gel point, i.e. at about 9 min, we found some small structures that were still moving with relatively high diffusion rates, up to 2.75 µm²/sec.

We found that diffusion coefficient calculations based on size and on velocity have the same range, 3.8 – 0.1 µm²/sec and were highly correlated, (r\textsuperscript{2} 0.87; p = 0.001). To determine the sizes of fibrin structures with such diffusion coefficients, we compared diffusion coefficients obtained from experimental data with those calculated for various sizes of protofibrils and fibers, i.e. aggregated protofibrils, with different diameters and lengths (for details see Materials and Methods section). At 1
min from the beginning of polymerization, the diffusion coefficients were in the range from 2.4 \( \mu \text{m}^2/\text{sec} \) to 3.8 \( \mu \text{m}^2/\text{sec} \). We found from the known fibrin structures that this range corresponded to only a few possible types of structures: protofibrils in the length range from 0.54 to 0.95 \( \mu \text{m} \), fibers consisting of two laterally aggregated protofibrils up to a length 0.67 \( \mu \text{m} \), and 3 laterally aggregated protofibrils up to a length of 0.62 \( \mu \text{m} \) (Fig. 6). Thus, the smallest structures we were able to see in the spinning disk confocal microscope from one minute after the beginning of polymerization until the gel point were protofibrils with a length of about 0.5 \( \mu \text{m} \).

**Discussion**

Early events of fibrin polymerization have been quantitatively studied by using different techniques, including turbidity\(^20\) light scattering\(^{21,22}\) microscopy\(^23\) and kinetic analyses\(^{12,18}\). They provide data from different standpoints, but all of these studies were based on indirect observations, rather than direct visualization, and yield average values. There was some direct observation of moving fibrin-structures at early stages of fibrin network formation\(^24\) but these images were not analyzed quantitatively because adequate technology was not available. We have used a different approach to solve this problem: electron microscopy, spinning disk confocal microscopy and turbidity measurements were synchronized. This is a powerful combination, since the strengths and weaknesses of these three techniques are complementary. Although spinning disk confocal microscopy allows the rapid real time imaging of hydrated structures, its resolution is limited, while electron microscopy requires dehydrated, stained specimens but allows high resolution visualization of small structures. Turbidity is a very commonly used technique that measures global properties of clots and can be used to characterize the kinetics as well as overall clot structure. In this paper, we provide quantitative analyses of fibrin-structures and the dynamics of changes of these structures prior to the gel point. We show that at the beginning of fibrin
polymerization, the population of fibrin-structures is not homogeneous and consists of monomers and small double-stranded structures, dimers, trimers, tetramers and protofibrils made from 8 or more monomers. These results are in agreement with light scattering data\textsuperscript{21,22} showing that during the lag period the population of fibrin structures is not homogenous but they were not able to characterize these structures in detail. Our data provide new information about the types of fibrin structures and the dynamic changes in proportions of these structures during the lag period and approaching the gel point. Structures made of 5, 6 and 7 monomers have not been observed in our studies, perhaps because these structures are not stable or the majority of small oligomers elongate rapidly.

At the middle of the lag period, the fraction of protofibrils was elevated compared to the fraction of dimers, trimers and fibers. This observation suggests that during this time the rate of longitudinal growth of oligomers or protofibrils is higher than the rate of lateral aggregation of these structures\textsuperscript{18}. At the time that turbidity began to rise, the percentage of fibers increased and the percentage of protofibrils decreased compared to the middle of the lag period, suggesting that the major process occurring at this time changed to lateral aggregation. Since the percentage of small structures, such as dimers, trimers and tetramers, did not differ much at the beginning and middle of the lag period, it could be that the rate of oligomer formation and rate of lateral aggregation were similar. The decrease of small structures at the end of the lag period could arise from their incorporation into bigger protofibrils or fibers. The lack of tetramers at the gel point could mean that their rate of association into larger structures is faster than for monomers, dimers and trimers. Small structures found at the gel point indicate that oligomer and network formation continues after the gel point, in agreement with our recently published results\textsuperscript{11}.

The diffusion coefficients we measured for moving structures that appear from the beginning of polymerization to the gel point decreased over this time because of increases in the size of these structures as a result of the processes of longitudinal and lateral growth and branching that are proceeding during this time. Although the diffusion coefficients for most structures dropped at the time when the turbidity curve started to rise, there were still some structures with high diffusion coefficients.
These findings support the results from our images discussed above that the population of fibrin structures is not homogeneous and that smaller structures are still present.

The sizes of the smallest structures observed in the spinning disk confocal microscope at one minute after the beginning of polymerization were determined from analysis of diffusion coefficients: protofibrils about 0.5 µm in length. This conclusion is consistent with the electron microscope images in that there were protofibrils and smaller structures but no fibers at 1 minute after initiation of polymerization (Fig. 3). Size measurement data of electron microscope images at the later time of polymerization of 3 minutes support this conclusion, because here the most frequent size was 0.4 µm, which corresponds to protofibrils built from 19 fibrin monomers. In other words, fibers are not the primary structures even at the middle of the lag period of fibrin polymerization.

In conclusion, the dynamic sequence of early events of fibrin clot formation from the beginning of polymerization to the gel point has been analyzed using the complementary techniques of electron microscopy, spinning disk confocal microscopy and turbidity measurements. Structures formed during the early stages of fibrin polymerization were visualized and dynamic changes in the proportion of these structures were characterized. The mobility and sizes of fibrin structures were characterized, and the smallest moving fibrin structures visible in a light microscope were identified. Understanding of the events responsible for gelation and their relation to the clotting process help us to interpret the clinical significance of changes in the gel point, which are widely used for assessment of coagulation in clinical laboratories. Knowledge of these aspects of the polymerization process may lead to new methods of prophylaxis, diagnosis, and treatment by modulation of specific steps that have been observed and analyzed here.

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Contribution: I.N.C designed the research and performed experiments, analyzed and discussed results, made figures and wrote the paper; C.N. carried out electron microscopy; and J.W.W. designed the research, discussed results, and wrote the paper.

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References


Figure Legends

Fig. 1. The initial part of the turbidity curve from the beginning of polymerization to the gel point. Turbidity curves were averaged from 3 identical experiments. The lag phase was from 0 to 345 ± 10 sec, and the gel point at 540 ± 11 sec. (▲) Arrowheads show the time when the polymerization reaction was stopped and samples were taken for transmission electron microscopy studies. (†) Arrow shows the gel point.

Fig. 2. Transmission electron micrographs showing representative fibrin structures. (A) monomers, (B) dimers, (C) trimers, (D) tetramers, (E) protofibrils, (F) fibers. (A-E) Magnification bar represents 50 nm. (F) Magnification bar represents 100 nm.

Fig. 3. Histograms showing the percentage of fibrin structures visualized by electron microscopy at 1, 3, 6, and 9 minutes during fibrin polymerization. All types of structures that appear in the images were counted in each micrograph. The number of monomers within each type of structure was calculated and normalized by the number of monomers present in the whole micrograph. (A) fibrin monomers, (B) dimers, (C) trimers, (D) tetramers, (E) larger oligomers and protofibrils, (F) fibers.

Fig. 4. Z projections of two spinning disk confocal micrographs at different times. This technique was used to measure the displacement of each individual fibrin structure during a known time interval to calculate the velocity.

(A) The displacement of a fibrin structure during 0.6 sec. (B) The displacement of another moving structure during 0.6 sec. (C) The projection of two Z sections, one 6 min and 5 sec and another one 6 min and 21 sec from the beginning of polymerization. (D) The projection of two Z sections, one at 6 min and 54 sec and another one 7 min and 5 sec. Arrows show the direction of movement. 1’ and 1”
correspond to one moving structure; 2’ and 2” correspond to another moving structure. Magnification bar represents 10 μm.

Fig. 5. Diffusion rates of fibrin structures calculated by two different methods as a function of time. ▲, calculations based on the sizes of moving structures; □, calculations based on the velocities of moving structures.

Fig. 6. Identification of fibrin structures by comparison of diffusion coefficients obtained from experimental data with diffusion coefficients calculated for model structures with different lengths and thicknesses. (A) diffusion coefficients obtained from experimental data; ▲, calculation based on size; □, calculation based on velocities of moving structures. (B) diffusion coefficient for ■ one protofibril; ○, two protofibrils; ♦, three protofibrils; ▽, four protofibrils; ●, five protofibrils. The dashed lines connecting A and B show the correlations between the measured (A) and calculated (B) diffusion coefficients, so it is possible to deduce what the structures observed could be (arrows at the right end of the dashed lines). For example, the top dashed line, for one of the largest diffusion coefficients measured, 3.8 μm²/sec, corresponds to a protofibril with a length of 0.54 μm. The lower dashed line is another example showing the possible fibrin structures that correspond to a diffusion rate of 2.4 μm²/sec, a single protofibril with a length of 0.95 μm, two laterally aggregated protofibrils with a length of 0.62 μm or three laterally aggregated protofibrils with a length of 0.67 μm.
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