Confocal reflectance microscopy and multiple particle tracking for the study of gelation of biopolymers

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ABSTRACT

The combination of confocal reflectance imaging (CRM) and simultaneous multiple particle tracking (MPT) with mean-square displacement (MSD) analysis is a new method for studying the gelation of biopolymers. In addition to showing the structure of the biopolymer-network by CRM, the microrheological properties at the same moment in gelation is shown by MSD-analysis of suspended particles. In this study the simultaneous use of these two methods has been used to investigate the fibrillogenesis and gelation of type 1 collagen. This new approach allows for non-invasive monitoring of the sample throughout the gelation process, revealing new insight on the correlation between network structures formed from during collagen self-assembly and the resulting mechanical properties of the gel.

INTRODUCTION

Confocal Reflectance Microscopy:

CRM is an optical imaging technique combining the intrinsic backscattering of light from biopolymers with confocal microscopy, to visualize the developing fibres. Collagen fibres will, like most other biopolymers, reflect light when reaching a large enough size. For a material to give a good reflection-contrast it needs to have a high reflectance or albedo, or show a significant index-of-refraction at it boundaries.[3] The coiled collagen fibres larger than 500nm diameter differ substantially in refractive index from a surrounding media like saline, reflecting light in the visual light spectrum.[5]

Confocal microscopes use point illumination, focusing on a small focal point in the sample. With a scanning laser different spots of the sample are illuminated in turn, regenerating a larger image. The reflected light is propagated back to the objective lens that focuses it into a spatial pinhole filter. Light from the illuminated spot in the correct focal plane is admitted, but light returning from out of focus planes is filtered out by the spatial pinhole. This allows viewing specifically a thin horizontal plane of interest, and mask out the planes above and below. This technique of imaging at a specific depth of the specimen is termed optical sectioning.[1] Resolution!

Combining reflectance- and confocal-methods allows for imaging of structures in the centre of a sample without the need for prior staining or physical slicing. The non-invasiveness of this procedure allows for monitoring of the same undisturbed location in a sample over time as the network develops. The real-time visualization of fibre-development, provides information on the kinetics of the self-assembly together with a detailed view of the structural dynamics during fibrillogenesis. This can be used for qualitative and quantitative evaluation of the structural parameters and organization of the developing fibre network.

Multiple-Particle Tracking and Mean-Square Displacement:

MPT is an analytical technique that allows

measurement of particle mobility in a solution. It is a high-throughput method of single-particle tracking (SPT), whereby measuring the motion pattern of multiple particles yields information on the overall particle-environment interactions. In this study, florescent spheres are added to the collagen solution prior to gelation, and particle motion at different timepoints throughout the sol-gel transition is analyzed.

Having captured the trajectories of particles, the particle-motion can be analysed. Mean-square displacement (MSD), is a measure of the displacement from reference position within a timescale.[2] For a particle trajectory tracked in two dimensions MSD is calculated by equation 1, combining the average displacement in both directions (Δx^2 and Δy^2). The positional change in each dimension is described as a function of time (t) and timescale (τ). Here x(t) and y(t) are the x- and y-coordinates of the particle at time t. τ is the timescale meaning the lag-time between the two frames chosen, as described in the previous section. The ensemble average MSD ($< MSD >$) is the mean of all trajectories from a single timepoint.

$$MSD = [x(t+\tau) - x(t)] + [y(t+\tau) - y(t)] \quad (1)$$

MSD as a function of timescale is used for motion type analysis by fitting log(MSD) and log(τ) to a straight line. When the displacement is linearly scalar to the timescale (MSD(τ) $\propto$ τ), the particles move by a purely diffusive motion. As shown in equation 2 and 3 the slope of the line called the anomalous exponent (α), indicates if the particles are diffusing freely $\alpha = 1$, or are undertaking anomalous subdiffusive motion $\alpha < 1$.

$$MSD = \tau^{\alpha} \times \Gamma \quad (2)$$

$$log(MSD) = \alpha \times log(\tau) + log(\Gamma) \quad (3)$$

During gelation α-values will naturally decrease, as the particles become increasingly confined by the collagen-network. When the solution is fluid(sol-state) the particles can diffuse freely, exhibiting brownian motion. When a gel is formed with a network mesh size comparable to particle-sizes, the particles become restricted in their motion. When the sol-gel transition has completed, a dense fibre-network will lead to full confinement of the particles, and there is elastic coupling between particles and network with the anomalous exponent approaching α=0.

EXPERIMENTAL

The collagen used is Cornings® type I rat tail collagen dissolved in 0.1% acetic acid (HAc), mixed with DPBS for a final 2mg/ml collagen concentration in the gels. Fibrillation is initiated upon neutralising the pH with 7.5%(w/w) sodium bicarbonate ($NaHCO_3$) and elevating temperatures to 37^{o}C. Invitrogen™ FluoSpheres® fluorescent particles is suspended in the solution prior to gelation. The particles are 0.5 μm diameter polystyrene microspheres beads with a carboxylated coupling surface, loaded with yellow-green fluorescent dye (Ex/Em = 505/515).

All imaging is performed with a confocal microscope Leica TCS SP8 using reflection- and fluorescence imaging. The Live Data Mode of the Leica LAS AF software allows for a predefined experiment "pattern" to be set up composed of multiple microscopy-"jobs". The pattern is set to take a single high resolution reflectance-image followed by a 20sec time-series of fluorescence-images with a framerate of 714ms. By "looping" the pattern, the same sequence of images is captured every 2.5 minutes for a total of 1h 30min.

The time-series of reflectance images are analyzed in ImageJ an open source Java image processing program, and MATLAB a scripting language used for numerical dataanalytics, developed by MathWorks. The pixel-intensities of reflectance images is used to estimate network-development. The time-series of time-lapses of the fluorescent particles are also analyzed in imageJ, using the plugin ParticleTrackerJ an algorithm made by Sbalzarini and Koumoutsakos[4] to identify and track par-

ticles. The particle trajectories are analyzed in MATLAB using the MATLAB per-value class "@msdanalyzer" developed by Tarantinoet al.[6]

RESULTS

An example of the confococal reflectance images is given in figure 1. The larger fibres are reflecting intensely, showing an interconnected network-structure. The florescent particles are visible as small bright spots, but do not interfere with the overall viewing of the collagen-fibres.

An example of the particle trajectories identified by MPT is given in figure 2. From left to right the particles show an increasingly confined motion, illustrating trajectories from start to end of collagen sol-gel transition.

Figure 3 shows the estimated MSD of particle-trajectories before, during and after gelation, at timepoints 2.5min, 30min and 80min respectively. This illustrates how during sol-gel transition the particles have increasingly confined motions, as the fibre-network surrounding them grows more dense and restricting. At 2.5min the particles exhibit a purely diffusive motion as no fibres can be

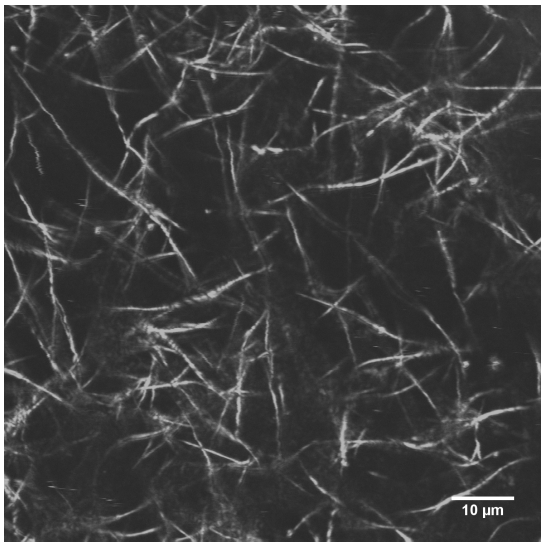

Figure 1. The fibre-network from self-assembly of 2mg/ml type I collagen after 1h 30min incubation at 37^{o}C.

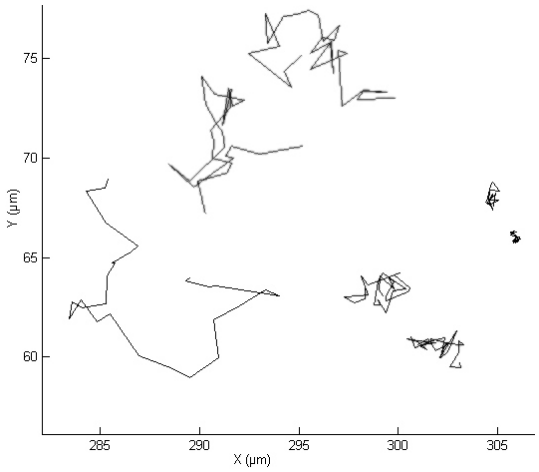

Figure 2. Trajectories of (0.5μm diameter polystyren microspheres with carboxylated surface) in 2mg/ml collagen solution.

detected in the CRM-images. AT 30min a few particles still appear to be moving freely, but the majority show and increasingly confined motion. At this time-point multiple fibres are visible in the CRM-images. At 80min CRM-images show a densely connected fibre-network of multiple fibres as seen in figure 1, this results in the near complete confinement of the florescent particles at the end of gelation.

Estimating the α-value from the ensemble-average MSD at each timepoint, the decline of the slope of the MSD-curves can be plotted. In figure 4 the α-values show that particles in the collagen sample start with a diffusive motion and become almost completely confined after 40min of gelation.

Finding the average pixel-intensity of the CRM-images from each timepoint gives a plot of intensity-increase throughout gelation. This can be interpreted as a measure of density of collagen fibre-network, where both growing number- and size- of fibres contribute to the increasing backscattering of light. The theoretical resolution of CRM is 200nm, while the smallest collagen fibres can have a diameter of only 30nm, meaning that early structures formed in the fibrillogenesis cannot be detected.

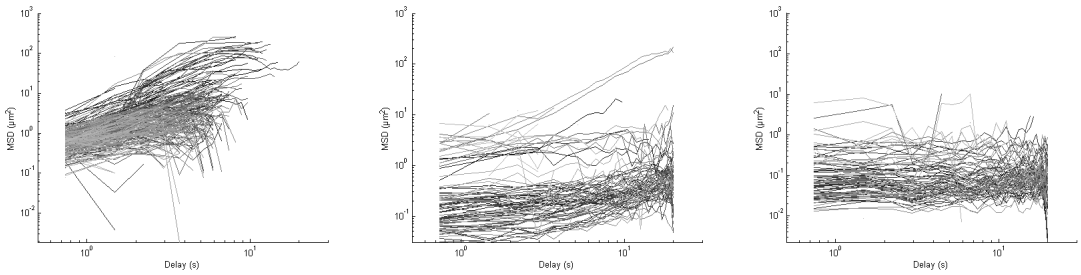

Figure 3. Logarithmic plots of MSD of particle-trajectories as a function of timescale τ at different timepoints. From left to right, 2.5min, 30 min, 80min after initialization gelation. (0.5μm diameter polystyren microspheres with carboxylated surface) in 2mg/ml collagen solution.)

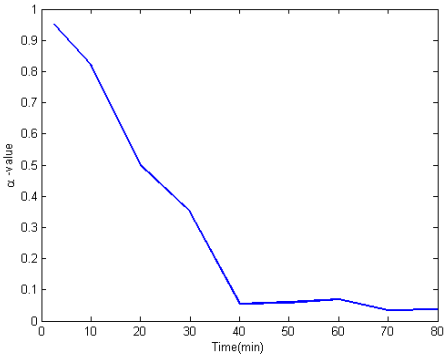

Figure 4. Anomalous exponent (α) from MSD-analysis of particle trajectories, plotted as a function of time. Decreasing motion of 0.5um carboxylated particles suspended in collagen solution gelling for 1h 30min at 37C.

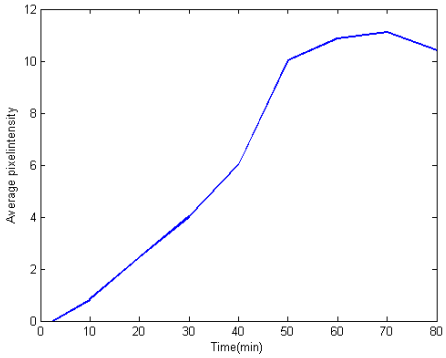

Figure 5. Pixelintensity in CRM-images of collagen fibres plotted as a function of time.

DISCUSSION

Comparing figure 4 of decreasing particle mobility and figure 5 of increasing fibre-network density, there is an obvious correlation between the two properties. The growth of the collagen fibre-network leads to confinement of the suspended particles. Since both measurements are done simultaneously on the same samples, direct comparison of microrheology as measured by MPT/MSD and structure as measured by CRM can be monitored throughout the sol-gel transition.

TL-CRM and MPT are both in principle non-invasive methods that do not influence the kinetics of the fibre-assembly. The driving forces in particle motion are to small to invoke sample deformation or disturbance of the microstructure. The fluorescent particles are visible in CRM images, as they reflect light at at the same wavelengths as the fibres. Yet their small size (500nm diameter) and relatively low number makes them only a minor disturbance when observing the collagen fibres.

The nanoparticles used for MPT are carboxylated, and have a negative surface-charge at neutral pH. This will affect the particle mobility, as they can interact with the positive charges on the collagen-network. Some particles are entrapped on or within fibres, and some are located in the fluid pores of the network. This means that we cannot distinguish between viscoelastic properties of the network elements or microrheology of the fluid within pores of the network, but rather obtain information on

the overall microrheological properties of the complex fluid that is the gel.

SUMMARY AND CONCLUSIONS

By simultaneous monitoring of both the assembly collagen fibre by CRM and the motility of the included particles, the collagen network-structure and the resulting viscoelastical properties can be measured throughout the sol-gel transition. Before gelation (sol-state) when no fibres are visible in CRM-images the particles move freely by diffusive motion. As the gelation progresses the collagen fibres increase in number and size forming an densely interconnected network structure. The suspended particles become increasingly confined by the surrounding network, exhibiting a restricted motion pattern.

Previous studies have performed individual microscopy and rheology experiments to study gelation, that would afterwards be compared to reveal the link between structural and viscoelastic properties of the assembling network. The results from these studies can therefor not be directly compared, but have to take into consideration the probable systematic errors. Since this experiment performs microrheology and reflectance imaging on the *same* samples at the *same* times, one can say with certainty that all parameters are identical for the the measurements. This enables direct comparison of microrheology and structure, providing an until now unachieved insight on the correlation between this properties in a gelling biopolymer system.

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