The influence of the sequence of nanoparticles injection to solution on the rate of fibrinogen-thrombin reaction

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Abstract. The influence of Fe$_2$O$_3$ nanoparticles on the rate of fibrinogen-thrombin reaction is studied. The nanoparticles were obtained in acoustoplasma discharge with cavitation. The sequence of nanoparticles injection appeared to change dramatically the rate and result of enzymatic reaction. In case of nanoparticles injection to fibrinogen before thrombin addition, enzymatic reaction practically stopped at the first stage. The mixing of nanoparticles with thrombin before its addition to fibrinogen leads to acceleration of gel formation in comparison with reaction without nanoparticles. We believe that Fe$_2$O$_3$ nanoparticles can modify the rate of enzymatic reaction, in one case acting as inhibitors of the reaction and as activators in other.

1. Introduction

Studies of the nanoparticles influencing on the biological systems are very important because of their wide application for different aims of modern medicine. They can be used for target and drug delivery, for visualization of metastasis, for stimulation of angi- and arteriogenesis, etc. Many papers are devoted to the investigations of nanoparticle interactions with proteins, for example, study of binding mechanism of Fe$_2$O$_3$ nanoparticles with fibrinogen by using a combination of circular dichroism, UV-vis fluorescence spectroscopic and computational methods [1]; or surface plasmon resonance study of fibrinogen and human serum albumin binding to metal oxide nanoparticles [2], etc. However, influencing of the nanoparticles on the rate of important biological reaction is not fully studied yet.

In our paper we investigated the influence of the sequence of Fe$_2$O$_3$ nanoparticles adding into solution on the rate of biological reaction of the fibrinogen cleavage by thrombin. This reaction is widely investigated by means of dynamic and static light scattering. For example, Kita et al in the paper [3] shows the time-dependence dynamic of the distributions of the scattered light intensity on particles sizes reflecting the fibrinogen-fibrin-gel transition under fibrinogen cleavage by thrombin. They determined time when the system begins to be a gel: at a gelation point the autocorrelation function $g^2(\tau)$ has no characteristic time and shows a power law behavior. Another big study of fibrin gels was conducted by Ferri et al [4] by elastic light scattering techniques and fractal analysis. They show that gels can be described as random networks of fibers of average diameter $d$ and density $\rho$, entangled together to form densely packed and spatially correlated blobs of mass fractal dimension $D_m$ and average size or crossover length $\xi$.

The aim of the study was to investigate dynamic of autocorrelation functions and distributions of scattered light intensity on particle sizes in fibrinogen-thrombin system without Fe$_2$O$_3$ nanoparticles and in case of different sequence of nanoparticles injection: (1) nanoparticles were added to fibrinogen
solution before injection of thrombin; and (2) nanoparticles were added to thrombin solution before adding this mixture to fibrinogen.

2. Methods and materials

For our study we used a traditional DLS setup with a He-Ne laser (the wavelength of 633 nm, the power of 5 to 10 mW), an optical system ensuring spatial coherence of recorded light, a goniometer allowing measurements at various angles, a special photomultiplier, a Photocor-FCm correlator (in the "Multiple tau" regime), and a PC with Photocor-FCm and DynaLS programs. For a more detailed description of the setup and DLS method see papers [5,6].

For model fibrinogen solution preparation we used fibrinogen from human plasma (Sigma Aldrich, ≥80% of protein is clottable) diluted in Tris-HCl buffer (7.2 pH). Final concentration of fibrinogen was about 3 mg/ml. After sample preparation solution was filtered through 0.2 μm “Superpure PVDF” filter.

Solution of thrombin was prepared using thrombin from human plasma (Sigma Aldrich, lyophilized powder, ≥2,000 NIH units/mg protein) that was diluted in Tris-buffer. Final activity of thrombin after adding it to fibrinogen solution was 1.7 NIH/mg. Mean hydrodynamic radius of the thrombin in a pure solution was about 230 nm.

Fe$_2$O$_3$ nanoparticles were obtained by unique acoustoplasma method with cavitation that combines effect of the elastic oscillations of high intensity ultrasound and pulsed or steady electric fields in a liquid medium. As a result relatively narrow size distribution of the synthesized nanoparticles with specific surface properties was obtained [7, 8]. Final volume concentration of Fe$_2$O$_3$ nanoparticles in model protein solution is $C_v \approx 0.5 \cdot 10^{-7}$ ($n_p \approx 4 \cdot 10^5$ cm$^{-3}$). Mean hydrodynamic radius of nanoparticles aggregates in the solution after ultrasound treatment was about 280 nm.

3. Results of experiment

3.1. Dynamic of distributions of scattered light intensity on sizes in fibrinogen-thrombin system.

First series of measurements was conducted in model solution of fibrinogen with thrombin. Figure 1a shows a distribution obtained for pure fibrinogen solution. In the distribution first peak having mean radius 13 nm and an area of the peak $A_1=0.728$ corresponds to fibrinogen monomers, second peak with mean radius 398 nm and area $A_2=0.049$ corresponds to essential aggregate of fibrinogen, as well as third peak (the area $A_i$ is logarithmic part of intensity, $I_i \sim A_i \cdot r_i$). Figure1 (b-f) shows temporal dynamic of distributions of scattered light intensity on particles sizes in the fibrinogen-thrombin system. Already through 20 min after thrombin addition, the distribution starts to change accordingly to enzymatic reaction of fibrinogen degradation, and after 40 min first peak size decreases from 13 nm to 2 nm as well as the area of first peak – from 0.728 to 0.028. Besides, area of second peak increases from 0.049 to 0.55.

The formation of fibrin gel is considered to have three main stages. In a first stage, cleavage of fibrinogen by thrombin at special sites leads to appearance of short peptides (radius and area decreasing of first peak in Figure1b-e) and fibrin in the solution; in a second stage – formation of protofibrills and their aggregate takes place (growth of mean radii of second and third peak in Figure1); and, finally, the whole network of fibrin fibers is forming in the solution (a set of radii appears in the distributions). These stages can overlap in time, how it can be seen in Figure 1.

In Figure 1 also presents values of amplitudes of correlation function of intensity $g^{(2)}(t)$ and the total intensities of scattered light (I/L). In gel formation before sol-gel transition the total intensity of scattered light (I/L) increases sharply, while the amplitude of correlation function decreases and the correlation function has such a shape, which gives a set of consecutive times or sizes being decomposed by exponents (Figures 1e,f). Such a picture appears already through 20-30 minutes after thrombin addition to pure fibrinogen solution (Figure 1b) and becomes more distinct in 1.5 hours. The maximal amplitude of correlation function is defined by geometry of experiment; and in case of liquid sample with Brownian particles (particle suspension) it equals to 0.35-0.4 in our geometry. One can
see that already through 20 minutes after thrombin addition (Figure 1b) the amplitude is equal 0.0315. Such a decreasing of this value indicates process of gel formation in the sample. In paper [3] authors pointed out that under approaching to sol-gel transition, scattering pattern has a form corresponding to fractal gel dimension and correlation function has a power law component; and the closer sample to gelled condition, the more this component.

![Figure 1](image)

**Figure 1 (a)** The distribution of the particles in pure fibrinogen solution; (b-f) dynamic of distributions of scattered light intensity on particles sizes in model fibrinogen-thrombin system; A1 – area of the peak, I/IL – scattered light intensity, Ampl. – amplitude of the autocorrelation function.

### 3.2. Dynamic of particle distributions in fibrinogen-thrombin system in case of Fe$_3$O$_5$ nanoparticle mixing with fibrinogen before thrombin addition

Second series of experiments was carried out with different sequence of Fe$_3$O$_5$ nanoparticles addition to model solution. First sequence was mixing of nanoparticles with fibrinogen before thrombin injection. Figure 2a shows distribution of fibrinogen with nanoparticles. As the fibrinogen particle distribution has the aggregates with mean radius 398 nm, the addition of nanoparticles Fe$_3$O$_5$ with mean radius 280 nm does not disturb the initial distribution significantly. Further injection of thrombin to fibrinogen-nanoparticle solution leads to unexpected result. Figures 2b-e show obtained dynamic of distributions of scattered light intensity on particles sizes. The distributions do not practically change: first peak has the unchanged mean radius and area (A$_1$) as well as second and third peak during more than one hour after thrombin addition. The total intensity of scattered light and the amplitude of correlation function vary slightly during experiment. It means that the presence of nanoparticle in the solution prevents thrombin to cleave fibrinogen, and further gel formation is difficult. Therefore, nanoparticles can play role of the inhibitors of enzymatic reaction.
Figure 2. (a) The distribution of particle sizes in solution of fibrinogen with nanoparticles, (b-e) dynamic of distributions of scattered light intensity on particles sizes in model solution in case of mixing of fibrinogen with Fe$_2$O$_3$ nanoparticles before thrombin addition to solution; Ai – area of the peak, I/IL – scattered light intensity, Ampl. – amplitude of the autocorrelation function.

3.3. Dynamic of particle size distributions in fibrinogen-thrombin system in case of Fe$_2$O$_3$ nanoparticle mixing with thrombin before its adding to fibrinogen solution

Second sequence of nanoparticle addition was carried out by mixing Fe$_2$O$_3$ nanoparticles with thrombin solution before its addition to fibrinogen solution (Figure 3). Figure 3b shows the distribution of scattered light intensity on sizes for solution of thrombin with nanoparticles: it has one peak with mean radius about 250 nm. Addition of thrombin-nanoparticle solution (Figure 3b) to fibrinogen solution (Figure 3a) leads to dramatically increasing of the total intensity of scattered light (from 800 a.u. to 400 000 a.u., i.e. almost in 500 times) and decreasing of amplitude of correlation function (from 0.41 to 0.042). Note that in case of enzymatic reaction without nanoparticles (Figure 1) the intensity jump was about 50 times. In case of thrombin-nanoparticle mixing, jump of the scattered light intensity is almost 500 times, which seems indicate enzyme reaction acceleration. Moreover, already through 30 min after solutions mixing distributions of scattered light intensity on radii have a set of consecutive times corresponding to autocorrelation functions with power law component (see Figures 3c, d). All these results point out fast gel formation in case of thrombin mixing with nanoparticles Fe$_2$O$_3$ before its addition to fibrinogen solution.
The distribution of scattered light intensity on particle sizes in pure fibrinogen solution; (b) the distribution for mixture of nanoparticles with thrombin, (c,d) dynamic of distributions of scattered light intensity on particles sizes in the solution in case of mixing of thrombin with Fe₂O₃ nanoparticles before injection to fibrinogen solution (gel formation), Aᵢ – area of the peak, I/IL – scattered light intensity, Ampl. – amplitude of the autocorrelation function.

Conclusions
In this paper we show that different sequence of Fe₂O₃ nanoparticles addition to model solution leads to absolutely different results. In case of mixing of fibrinogen with nanoparticles before thrombin addition, enzyme reaction is practically stopped at the first stage. In case of addition of nanoparticles to thrombin before its injection to fibrinogen, the reaction was accelerated and third stage of gel formation was registered already through 30 min after reaction initiation. We believe that nanoparticles can play role of inhibitors or activators of enzymatic reaction depending on the sequence of their addition. In first sequence of addition, they could cover the fibrinogen sites binding to thrombin, thereby preventing the interaction of the protein with enzyme. In second sequence of nanoparticle injection, interaction of nanoparticles with thrombin activates the enzyme and leads increasing of the reaction rate.

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