Spectral and structural properties of clotting factor proteins under mechanical stress

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Abstract. The fluorescence data obtained from BODIPY molecular sensors prove the increase of hydrophobicity of blood plasma proteins, fibrinogen, after in vitro and in vivo pressure episodes, which indicates conformational changes in the structure of the fibrinogen due to the phase transition from α-helices to β-sheets in its superspirals. The phase transition in the supramolecular structure of fibrinogen provides greater hydrophobicity of fibrin, intensifying the visible crystallinity of the fibrin clot observed by infrared spectroscopy, scanning electron microscopy and X-ray imaging.

1. Introduction

The federal project of Russian Federation to fight cardiovascular diseases is aimed at developing and implementing innovative medical technologies including an early detection system and remote patient monitoring. The basis of these socially significant diseases, which are currently poorly studied and characterised by low predictability, are disorders of the aggregate state of the blood. The system of regulation of its aggregate state is a hemostatic potential, which is formed by a complex of proteins-enzymes that are selectively involved in the clotting process. Interactions between enzymes and substrates depend on factors affecting their properties.

A series of papers in clinical medicine have investigated changes in the structure of blood proteins due to their possible liquid-crystalline properties [1,2].

The crystalline state of fibrin, the main protein of blood thrombosis, was studied in [3,4]. It was proved that mechanical influences on fibrin clot (both stretching and compression) lead to conformational changes in the protein: the so-called phase transition from α-helices to β-sheets in fibrin superspirals occurs, which results in changes in its secondary structure and, as a consequence, bioactivity.

The structure of fibrin is quite obviously conditioned by the structure of its precursor - fibrinogen - blood coagulation factor I. Therefore, it is rational to assume that the effect of pressure on liquid plasma containing fibrinogen causes conformational changes in the structure of its molecules due to the replacement of α-helical sites by β-conformation. This leads to changes in the physicochemical characteristics of the polymerization process of fibrinogen into fibrin, underlying blood coagulation.

To prove this assumption, we used an in vitro model of static loading of the platelet-free plasma.
The most sensitive and informative instrumental methods to study the processes in biological media with biomolecules include, for example, electron microscopy, infrared spectroscopy and spectrofluorimetry, in which fluorescent molecular rotors based on BODIPY complexes [5,6] have been well established.

2. Experiment
Blood plasma without hemolysis traces was used for the study. Venous blood of volunteers (men aged 18 to 25 years) was taken into a plastic tube containing 3.8% sodium citrate solution (9:1). It was stirred and centrifuged for 15 min at 3000 rpm. The supernatant (plasma) was collected and used for research.

1 ml of plasma was placed in a sterile 10 ml sterile disposable syringe. The syringe was tightly sealed by placing it in paraffin (h). The syringe was fixed in a rack (figure 1). A system of weights was placed on the piston of the syringe, creating a static pressure on the plasma. To calculate the force application (N) and obtain the necessary pressure on blood plasma, the data in table 1 were used.

![Figure 1. Schematic of the pressure unit.](image)

One cycle of the experiment required 1 ml of plasma, which was divided into 4 equal portions. The first portion was a control and was pumped into a syringe and placed vertically in a rack for 60 minutes. The second portion was subjected to mechanical impact of pressure, approximately corresponding to systolic blood pressure in arterial vessels, 113 mmHg, which was generated by a system of weights placed on the syringe piston for 60 minutes. The third batch was mechanically exposed to a higher than normal systolic blood pressure of 189 mmHg, which was also generated by 60 minutes of weights on the syringe piston. The fourth portion was subjected to a mechanical pressure roughly corresponding to the hypertensive blood pressure in the arterial vessels - 264.74 mmHg also for one hour.

Electronic absorption spectra (in the range of 350-650 nm) and fluorescence spectra (in the range of 350-650 nm) were recorded on spectrofluorimeter Solar SM2203 (Belarus). To determine the characteristics of fluorescence we used 480 nm wavelength. Width of excitation and emission slits was 5 nm. All experiments were performed in a thermostatically controlled cell at 37 °C.

A compound based on bordipyrrine complexes (BODIPY) 4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-phenyl-4-boro-3a,4a-diaza-indacene, which has sufficient selectivity for the action of blood plasma proteins, was used as fluorophore [1]

To register the fluorescence spectrum of this dye, 0.4 ml of plasma, 2.45 ml of water and 0.15 ml of BODIPY-fluorophore solution in DMSO (C_{BODIPY} = 10^{-5} mol/L) were added to a thermostable spectrofluorimeter cell and mixed.

After recording the electronic fluorescence spectrum, the coagulation process was started by adding 0.04 mL of 1% calcium chloride to the cuvette.

After the addition of calcium chloride at regular intervals, the fluorescence intensity at the maximum emission of BODIPY - fluorophore was measured automatically using a spectrofluorimeter, recording the coagulation curve of the plasma sample. The study was stopped when a steady state plateau in the
fluorescence curve was reached. Fibrin clot (white clot) obtained in the cuvette was carefully laid out on KRS-glass without mechanical deformation and dried at room temperature to a dry film for infrared spectroscopy, electron microscopy and radiography.

X-ray diffractometer D2 PHASER from Bruker with CuKα-radiation (λ = 1.5406 Å) with a step of 0.0200 and focusing geometry by Bragg-Brentano with fixed module of primary slit and linear position-sensitive counter LYNXEY of the center for joint use of scientific equipment “The upper Volga region centre of physico-chemical research” was used for the X-ray diffraction study of samples.

Electron microscope EM Tescan 3 SBH was used for visual control of changes in blood plasma protein structures. IR - spectra were recorded on Fourier spectrometer TENSOR-27 Bruker Optics (Center for Shared Use of Scientific Equipment of the ISUCT, Ivanovo).

3. Results and discussion

It was found [1, 2] that fibrinogen is capable of forming molecular complexes with BODIPY fluorophores, and it was shown that for hydrophobic compounds like 4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-phenyl-4-boro-3a,4a-diaza-indacene, complexation occurs at hydrophobic sites of protein macromolecules. This is directly related to the quantum yield values of BODIPY fluorescence.

Figure 2 shows the electronic fluorescence spectra of the dye 4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-phenyl-4-boro-3a,4a-diaza-indacene in a solution of platelet-free plasma before pressure, after exposure to 113 mmHg (3 N) and after exposure to 189 mmHg (5 N).

![Figure 2](image-url)

**Figure 2.** Fluorescence spectra (BODIPY) of 4,4-difluoro-1,3,5,7-tetramethyl-2,6-diethyl-phenyl-4-boro-3a,4a-diase-indacene with blood plasma.

From the data of figure 2 we can conclude that the following changes occur in the electronic fluorescence spectra:

- Increase in integral fluorescence intensity in plasma under 113 mmHg (3 N) pressure. (3 N) compared to control plasma. The increase of quantum yield of fluorescence relative to the original plasma is 9%;
- Increase of integral fluorescence intensity in plasma under 189 mmHg (5 N) compared to control plasma. (5 N) compared to plasma samples, both initial and under 113 mmHg load. (3 H). The increase in quantum yield of fluorescence relative to the original plasma is 12%.

The obtained spectral characteristics of BODIPY showed an increase in hydrophobicity of blood plasma proteins, in particular, fibrinogen, after episodes of pressure rise in vitro. This may indicate conformational changes in the structures of sampled proteins due to the phase transition from α-helices to β-sheets in its superspirals. The phase transition in the supramolecular structure of fibrinogen causes greater hydrophobicity of fibrin, enhancing the visible crystallinity of the fibrin clot observed by scanning electron microscopy (figure 3).
Infrared spectroscopy showed (figure 4) that the effect of pressure is accompanied by a change in the spectrum of amide I, which is typical for an increase in the proportion of β-structures [3]. Amide 1 of healthy donor plasma without pressure effects has a characteristic absorption maximum at 1654.5 cm⁻¹. The maximum at this wavelength is typical and is registered if α-helices predominate in the conformational structure of the protein and the proportion of β-sheets in the superspirals is negligible. Exposure to 5 H for 60 min leads to an absorption maximum of 1625 cm⁻¹, which is characteristic of the antiparallel β-structures [7,8] formed in the process of protein aggregation upon unfolding of α-helices. This enhances the hydrophobic properties of the biopolymer. Hydrophobic interactions displace water from peri- and inter-protein space, increasing protein crystallinity, as shown in figure 3.
Figure 4. Primary IR spectra of fibrin amide band 1. 1 - obtained without additional influence; 2 and 3 - under overpressure of 189 mmHg. (5 N) on two different plasma samples.

X-ray diffraction method makes it possible to determine not only the content of the crystalline phase, but also that of the amorphous phase. The content of the amorphous phase is determined either by the difference of one and all crystalline phases (in fractions), or independently. In the second method it is taken into account that the coherent X-ray scattering intensity of the amorphous phase is proportional to its content. Figure 5 shows X-ray pictures of fibrin films obtained from plasma which was not subjected to additional pressure and plasma after loading.

Both methods are used only for a comparative assessment of the proportion of the amorphous phase. Crystallinity index was calculated for the investigated samples (using DIFFRAC.EVA package), the data are given in table 1.

Table 1. Crystallinity indices of samples of fibrin films (white clot) obtained from human plasma subjected to pressure for one hour.

| №  | Load (pressure) | %-Crystallinity | %-Amorphous |
|----|----------------|-----------------|-------------|
| 1  | 0 N            | 46.0            | 54.0        |
| 2  | 3 N (113.46 mmHg) | 48.2            | 51.8        |
| 3  | 7 N (189.09 mmHg) | 53.1            | 46.9        |

The data in this table also prove that exposure to pressure has an effect on the plasma, increasing the crystallinity of the fibrin clot.

Figure 5. X-ray diffraction data of fibrin protein films of plasma after pressure influence. (a) - without additional influence, (b) - after influence of overpressure of 264.74 mmHg. (7 N) for 60 min.
4. Conclusions
Fluorescence spectroscopy revealed that in the system consisting of BODIPY and blood plasma subjected to 113 mmHg (3 N), there is a 9% increase in quantum yield of fluorescence, while when blood plasma is exposed to 189 mmHg (5 N) there is an increase of fluorescence quantum yield by 12%. This effect may indicate an increase in hydrophobicity of blood plasma proteins as a result of conformational changes in fibrinogen.

The method of infrared spectroscopy shows a change in the spectrum of amide I, which is characterized by an increase in the proportion of β-structures, which also indicates an increase in the hydrophobic properties of the biopolymer and displacement of water from the interprotein and near-protein space.

X-ray examination of dry fibrin clot films with Bragg-Brentano focusing geometry and subsequent calculation of the crystallinity index indicate a 2.2% increase in crystallinity of samples subjected to mechanical loading in the case of 113 mmHg pressure (3 N) and 7.1 for 189 mmHg (5 N).

Studies using scanning electron microscopy also showed a visible increase in crystallinity in samples of platelet-free plasma subjected to mechanical stress, as well as platelet-rich plasma.

The results of our experiment can show that the mechanical impact on blood plasma, leading to an increase in static pressure, affects the structural properties of proteins: fibrinogen macromolecules change their secondary structure, switching from α-helices to β-sheets in its superspirals.

The phase transition in the supramolecular structure of fibrinogen causes greater hydrophobicity of fibrin, increasing crystallinity of the fibrin clot, which may be the basis (cause) of changes in the enzymatic activity/functional properties of the blood coagulation system.

The results of the study can be used in the framework of the federal project "Combating cardiovascular diseases" to develop laboratory methods for cardiovascular risk assessment.

Acknowledgements
The study was performed in the frames of the state task of the Ministry for Healthcare of the Russian Federation №056-00028-2100 "Functional reserve of hemostasis and blood rheology in hypoxic states in norm and pathology".

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