VIA MEDICA

ORIGINAL ARTICLE

Joanna Sikora[1], Małgorzata Ostrowska[2], Michał Kasprzak[1, 3], Jacek Fisz[4], Grażyna Odrowąż-Sypniewska[5], Jacek Kubica[1]

[1]Department of Cardiology and Internal Medicine
[2]Department of Principles of Clinical Medicine
[3]Department of Pharmacology and Therapeutics
[4]Department of Laser Therapy and Physical Therapy
[5]Department of Laboratory Medicine
Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland

Influence of deep-freezing on the autofluorescent properties of human plasma proteins

Corresponding author:

Jacek Kubica, Department of Cardiology and Internal Medicine, Nicolaus Copernicus University, Collegium Medicum, Skłodowskiej-Curie Street No. 9, 85–094 Bydgoszcz, Poland
E-mail: jkubica@cm.umk.pl

Folia Medica Copernicana 2014; Volume 2, Number 1, 19–25

ISSN 2300–5432

ABSTRACT

Background. Fluorescence-based techniques are powerful and valuable tools for studying biological materials. Fluorescence spectroscopy is one of these techniques which measures autofluorescence from a sample containing components such as proteins. The aim of this study was to evaluate the influence of deep freezing on spectroscopic properties of human plasma using fluorescence spectroscopy.

Patients, materials and methods. The study group consisted of patients admitted to the Department of Cardiology and Internal Medicine at the University Hospital in Bydgoszcz, due to a preliminary diagnosis of acute coronary syndrome. The overall group comprised 27 patients. From each patient, 4 ml of blood was taken to obtain plasma that was used for further examination. In order to measure plasma spectroscopic properties, a Hitachi F-7000 spectrofluorimeter was used. The received results were analysed and evaluated with Origin 9.0 (OriginLab).

Results. Spectroscopic analysis of human plasma before and after freezing allowed us to divide plasma samples into three different subgroups, depending on their fluorescent properties. The first subgroup consisted of plasma samples, which showed entirely differently in spectroscopic analysis after one week of deep-freezing. The second subgroup of plasma samples showed partial changes in the measurements of autofluorescence, and in the third subgroup freezing-resistant plasma samples were included.

Conclusions. It seems that the process of deep-freezing could affect the autofluorescent properties of human plasma proteins. The explanation of the specific mechanisms responsible for the change of plasma fluorescent properties during the process of deep-freezing requires further elucidation.

Key words: autofluorescence, proteins, deep-freezing, human plasma, fluorescence spectroscopy

Folia Medica Copernicana 2014; 2 (1): 19–25

Introduction

Fluorescence-based techniques are powerful and valuable tools for studying biological materials. They provide information on the structure of biomolecules and the mechanism of their action. Fluorescence spectroscopy is one of the fluorescence-based techniques which measures fluorescence from a sample containing components such as proteins, nucleic acids and other substances which can emit autofluorescence. Proteins have the largest contribution in the effect of autofluorescence in biological materials. Human plasma consists of approximately 700 core proteins, as well as their precursors, metabolites, their glycosylated and phosphorylated forms, and the products of post-translational modifications. This indicates that the plasma proteome contains several dozen thousands of molecular forms of classic proteins. Hence, the protein profile (proteome) may differ depending on the metabolic pathways, individual features, and different environmental conditions.

In their pioneering work, Teale et al. reported that proteins are capable of emitting luminescence after excitation by ultraviolet light. The fluorescence properties of proteins are connected to the presence of aromatic amino acids in their polypeptide chains, such as tryptophan, tyrosine and phenylalanine [1, 2]. Tryptophan is the dominant fluorophore of the proteins despite the small number of its residues in proteins,

which is caused by the high level of metabolic expense of its synthesis. Changes in the emission spectra of tryptophan often occur as a consequence of subunit association, conformational transitions, substrate binding and denaturation [3, 4].

Deep-freezing (at –80°C) of human plasma is a common storage method, even for a long time, assuming that at such a low temperature all biochemical processes are essentially reduced or inhibited. The aim of this study was to evaluate the influence of deep-freezing on autofluorescent properties of human plasma by means of fluorescence spectroscopy.

Patients, materials and methods

Study group

The study group consisted of patients admitted to the Department of Cardiology and Internal Medicine in University Hospital no 1 in Bydgoszcz due to a preliminary diagnosis of acute coronary syndrome. Every patient received thorough information about the study and gave their written consent. The overall group comprised 27 patients, mean age 61 ± 14 years — 22 men (81.5%), mean age 59.6 ± 14.1 years, and five women (18.5%), mean age 64.6 ± 14.2 years. From each patient, a 4 ml blood sample was taken for examination.

Methods

In order to measure plasma spectroscopic properties, a Hitachi F-7000 spectrofluorimeter was used. The measurements were made only in specified conditions which were determined on the basis of our earlier preliminary experiments. Shortly afterwards, comprehensive measurements using human plasma were performed. Then, conditions were chosen at which the autofluorescence was the highest and the most characteristic. The obtained results were analysed and evaluated with Origin 9.0 (OriginLab).

Measurement procedure

The plasma was obtained by spinning fresh blood collected in the tubes containing EDTA in standard conditions. Plasma samples were aliquoted in two small tubes. In the first sample, the spectroscopic measurements were performed within three hours. The second plasma sample was frozen at –80°C for more than one week. Thawing of samples was performed in a few steps according to good laboratory practice. In the first stage, the plasma was placed in –20°C for 0.5 h, thereafter in 4°C for 0.5 h. Plasma samples were kept at room temperature for 0.5 h before performing the measurements. For the purpose of spectroscopic measurements, 300 μL of plasma was taken.

The autofluorescence signals of the thin layers (2 mm) of plasma specimens, placed between two quartz plates, were measured in the spectrofluorimeter at particular experimental geometry and at spectroscopic conditions, selected from independent (preliminary) measurements. The autofluorescence emission and excitation spectra were collected at front-face excitation-detection conditions, i.e. the same side of the sample was probed in the autofluorescence excitation and detection processes. The angle between the normal to the sample and the direction of exciting light propagation was set to about 60° in order to reduce the intensity of the scattered exciting light. The emission spectra were collected at the excitation wavelengths λ = 320 nm, λ = 340 nm, λ = 360 nm, λ = 380 nm, λ = 400 nm, λ = 450nm, while the excitation spectra were measured at the fluorescence detection wavelengths λ = 380 nm, λ = 400 nm, λ = 430 nm, λ = 450 nm, λ = 500 nm.

Statistical analysis

According to the Shapiro-Wilk test, the investigated continuous variables were non-normally distributed, therefore, they were reported as medians and interquartile ranges (IR). For comparisons between two groups, the Mann-Whitney unpaired rank sum test was used. Categorical variables were expressed as a number of patients presenting the given feature and a percentage of patients in the analysed group. Categorical variables were compared using the χ^2 test with the Yates' correction or Fisher's exact test depending on the number of observations. Differences were considered significant at $p < 0.05$. P values of ≥ 0.05 and < 0.10 were considered as a trend towards significance. The statistical analysis was carried out using the Statistica 10.0 package (StatSoft, Tulsa, OK, USA).

Results

Spectroscopic analysis of the human plasma samples before and after freezing allowed us to split the patients into three different groups with respect to the differences in autofluorescence emission spectra (subgroups A, B and C), and also into three different groups with respect to the differences in autofluorescence excitation spectra (subgroups A', B' and C') (Tabs. 1 and 2). Taken together, two sets of three subgroups of autofluorescence spectra were distinguished. The clinical characteristics of patients from all six subgroups are presented in Table 3.

The first subgroups (A and A') of both sets consisted of plasma samples which featured entirely differently in spectroscopic analysis after one week of deep-freezing. By 'entirely different' spectroscopic properties, we mean:

Table 1. Emission spectra: A — group of the plasma samples, which were signalised entirely differently after deep freezing, B — group of the plasma samples, which were signalised partially differently after deep freezing, C — group of the plasma samples, which were freezing-resistant

A	1 2 9 12 21	19%
B	5 7 11 17 18 19 20 22 23 25 26 27	44%
C	3 4 6 8 10 13 14 15 16 24	37%

Table 2. Excitation spectra: A' — group of the plasma samples, which were signalised entirely differently after deep freezing, B' — group of the plasma samples, which were signalised partially differently after deep-freezing, C' — group of the plasma samples, which were freezing-resistant

A'	5 6 7 8 9 10 14 15 16 17 18 22 23 26 27	55%
B'	1 2 3 12 13 19 20 21	30%
C'	4 11 24 25	15%

Table 3. Clinical characteristics of the whole group and comparison of the three subgroups with respect to the differences in autofluorescence emission spectra

Clinical characteristics	Whole group n = 27	A n = 5 (18.5%)	B n = 12 (44.4%)	C n = 10 (37.0%)	p (A–B)	p (B–C)	p (A–C)
Men	22 (81.5%)	4 (80.0%)	8 (66.7%)	10 (100.0%)	NS	**0.963**	NS
Age	63.0 (49.0–72.0)	49.0 (48.0–61.0)	63.0 (49.0–76.0)	59.0 (53.0–67.0)	NS	NS	NS
Confirmed ACS	22 (81.5%)	5 (100.0%)	8 (66.7%)	9 (90.0%)	NS	NS	NS
HyperteNSion	16 (59.3%)	3 (60.0%)	7 (58.3%)	6 (60.0%)	NS	NS	NS
Diabetes mellitus	9 (33.3%)	1 (20.0%)	3 (25.0%)	5 (50.0%)	NS	NS	NS
Dyslipidaemia	11 (40.7%)	2 (40.0%)	5 (41.7%)	4 (40.0%)	NS	NS	NS
Smoking	16 (59.3%)	3 (60.0%)	6 (50.0%)	7 (70.0%)	NS	NS	NS
Heart failure	10 (37.0%)	1 (20.0%)	5 (41.7%)	4 (40.0%)	NS	NS	NS
Previous ACS	7 (25.9%)	1 (20.0%)	3 (25.0%)	3 (30.0%)	NS	NS	NS
Leukocytes (G/l)	10.1 (7.0–13.6)	9.2 (7.8–10.1)	9.6 (6.4–11.9)	12.7 (10.3–14.0)	NS	NS	NS
Erythrocytes (T/l)	4.7 (4.5–4.9)	4.9 (4.9–5.2)	4.5 (4.3–4.7)	4.8 (4.6–5.0)	**0.051**	**0.081**	NS
Haemoglobin (g/dl)	14.0 (13.0–15.0)	15.2 (14.9–15.2)	13.7 (12.1–14.3)	14.1 (13.8–15.0)	**0.004**	**0.059**	NS
Haematocrit (%)	42.0 (40.0–44.0)	44.8 (44.1–45.7)	40.9 (37.2–42.7)	41.9 (40.7–44.6)	**0.0097**	NS	NS
MCV (fl)	89.0 (87.0–92.0)	90.7 (88.7–91.6)	90.7 (85.1–93.5)	88.0(87.3–91.2)	NS	NS	NS
MCH (pg)	30.0 (29.0–31.0)	30.7 (29.5–31.5)	30.7 (28.7–31.4)	29.8 (28.7–30.1)	NS	NS	NS
MCHC (g/dl)	33.4 (32.2–33.9)	33.8 (33.3–34.4)	33.4 (32.9–33.8)	33.5 (32.7–33.7)	NS	NS	NS
Platelets (G/l)	208 (159.0–237.0)	208.0 (159.0–237.0)	204.0 (156.5–237.0)	212.0 (195.0–236.0)	NS	NS	NS
RDW-SD (fl)	45.0 (43.0–46.0)	44.7 (43.0–46.0)	43.0 (41.2–45.4)	45.5 (43.7–46.1)	NS	NS	NS
RDW–CV (%)	14.0 (13.0–14.0)	13.6 (13.5–14.1)	13.5 (13.1–14.1)	14.0 (13.4–14.5)	NS	NS	NS
PDW (fl)	13.0 (12.0–15.0)	14.1 (11.7–15.0)	13.8 (12.4–14.7)	12.7 (12.2–13.1)	NS	NS	NS
MPV (fl)	11.0 (11.0–12.0)	11.6 (10.1–12.0)	10.9 (10.4–11.5)	10.8 (10.5–10.9)	NS	NS	NS
P-LCR (%)	32.0 (29.0–36.0)	36.7 (26.6–39.0)	32.8 (28.6–35.6)	30.5 (28.6–32.1)	NS	NS	NS
PCT (%)	0.2 (0.2–0.3)	0.3 (0.2–0.3)	0.2 (0.2–0.3)	0.2 (0.2–0.3)	NS	NS	NS
Total cholesterol (mg/dl)	173.5 (151.0–222.0)	198.0 (152.0–244.0)	153.0 (147.0–197.0)	181.0 (165.0–239.0)	NS	NS	NS
HDL cholesterol (mg/dl)	48.0 (37.0–56.0)	53.0 (50.0–64.0)	41.0 (35.0–56.0)	44.0 (40.0–56.0)	NS	NS	NS
Triglycerides (mg/dl)	105.5 (66.0–128.0)	147.0 (66.0–157.0)	85.0 (64.0–123.0)	106.0 (79.0–136.0)	NS	NS	NS
LDL cholesterol (mg/dl)	113.0 (78.0–150.0)	125.0 (76.0–168.0)	96.0 (72.0–135.0)	118.0 (99.0–168.0)	NS	NS	NS
Troponin I (ng/ml)	0.7 (0.1–8.5)	0.3 (0.1–1.0)	1.0 (0.1–8.5)	2.2 (0.1–12.2)	NS	NS	NS
Creatinine (mg/dl)	0.9 (0.8–1.3)	0.8 (0.8–0.8)	0.9 (0.8–1.2)	1.0 (0.8–1.3)	NS	NS	NS
Sodium (mmol/l)	140.0 (139.0–141.0)	139.8 (139.8–140.6)	140.8 (139.0–143.2)	140.0 (138.1–141.1)	NS	NS	NS
Potassium (mmol/l)	5.0 (4.0–5.0)	4.8 (4.3–5.0)	4.4 (3.8–4.6)	4.4 (3.7–4.6)	NS	NS	NS
Chloride (mmol/l)	104.0 (103.0–108.0)	104.5 (104.2–105.8)	105.6 (103.8–108.4)	103.6 (100.1–109.7)	NS	NS	NS
Glucose (mg/dl)	110.5 (99.0–154.0)	106.5 (94.0–127.0)	109.0 (100.0–128.0)	151.5 (91.0–159.0)	NS	NS	NS

ACS — acute coronary syndrome; HDL — high density lipoprotein; INR — international normalised ratio; LDL — low density lipoprotein; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration; MCV — mean corpuscular volume; MPV — mean platelet volume; NS — non significant; PCT — plateletcrit; P-LCR — platelet large cell ratio; PDW — platelet distribution width; RDW-CV — red blood cell distribution width coefficient variation; RDW-SD — red blood cell distribution width standard deviation

Table 4. Clinical characteristics of the whole group and comparison of the three subgroups with respect to the differences in autofluorescence excitation spectra

Clinical characteristics	Whole group n = 27	A' n = 15 (55.6%)	B' n = 8 (29.6%)	C' n = 4 (14.8%)	p (A'–B')	p (B'–C')	p (A'–C')
Men	22 (81.5%)	11 (73.3%)	7 (87.5%)	4 (100.0%)	NS	NS	NS
Age	63.0 (49.0–72.0)	54.0 (45.0–70.0)	62.0 (51.0–76.0)	67.5 (65.0–70.0)	NS	NS	NS
Confirmed ACS	22 (81.5%)	10 (66.7%)	8 (100.0%)	4 (100.0%)	NS	NS	NS
Hypertension	16 (59.3%)	8 (53.3%)	5 (62.5%)	3 (75.0%)	NS	NS	NS
Diabetes mellitus	9 (33.3%)	5 (33.3%)	2 (25.5%)	2 (50.0%)	NS	NS	NS
Dyslipidaemia	11 (40.7%)	6 (40.0%)	3 (37.5%)	2 (50.0%)	NS	NS	NS
Smoking	16 (59.3%)	10 (66.7%)	5 (62.5%)	1 (25.0%)	NS	NS	NS
Heart failure	10 (37.0%)	5 (33.3%)	2 (25.5%)	3 (75.0%)	NS	NS	NS
Previous ACS	7 (25.9%)	5 (33.3%)	0	2 (50.0%)	NS	NS	NS
Leukocytes (G/l)	10.1 (7.0–13.6)	10.1 (7.0–13.6)	11.4 (8.3–13.3)	9.1 (5.7–13.2)	NS	NS	NS
Erythrocytes (T/l)	4.7 (4.5–4.9)	4.6 (4.5–4.9)	4.7 (4.5–5.0)	4.9 (3.8–5.0)	NS	NS	NS
Haemoglobin (g/dl)	14.0 (13.0–15.0)	14.0 (12.1–14.7)	14.6 (13.9–15.0)	14.0 (11.1–14.6)	NS	NS	NS
Haematocrit (%)	42.0 (40.0–44.0)	41.7 (37.9–43.3)	43.8 (40.9–45.0)	41.5 (34.0–43.1)	NS	NS	NS
MCV (fl)	89.0 (87.0–92.0)	89.4 (86.4–92.2)	91.0 (88.2–91.8)	86.4 (84.0–95.3)	NS	NS	NS
MCH (pg)	30.0 (29.0–31.0)	30.1 (28.7–31.0)	30.3 (29.2–31.2)	29.2 (28.3–31.0)	NS	NS	NS
MCHC (g/dl)	33.4 (32.2–33.9)	33.5 (33.3–34.2)	33.3 (32.7–33.8)	33.5 (32.4–33.8)	NS	NS	NS
Platelets (G/l)	208 (159.0–237.0)	207.0 (178.0–255.0)	211.5 (153.5–234.5)	191.0 (153.0–228.5)	NS	NS	NS
RDW-SD (fl)	45.0 (43.0–46.0)	43.1 (41.6–45.5)	45.7 (43.8–47.1)	49.6 (43.3–54.8)	NS	NS	**0.035**
RDW-CV (%)	14.0 (13.0–14.0)	13.4 (13.1–14.2)	14.0 (13.6–14.4)	15.0 (13.9–16.2)	NS	NS	NS
PDW (fl)	13.0 (12.0–15.0)	12.8 (12.2–13.8)	15.0 (13.0–15.0)	12.9 (11.5–15.4)	**0.071**	NS	NS
MPV (fl)	11.0 (11.0–12.0)	10.7 (10.5–11.0)	11.6 (10.9–11.9)	10.9 (10.2–12.0)	**0.0109**	NS	NS
P-LCR (%)	32.0 (29.0–36.0)	30.2 (28.3–33.0)	36.3 (32.1–40.0)	31.3 (26.2–38.9)	**0.016**	NS	NS
PCT (%)	0.2 (0.2–0.3)	0.2 (0.2–0.3)	0.3 (0.2–0.3)	0.2 (0.2–0.2)	NS	NS	NS
Total cholesterol (mg/dl)	173.5 (151.0–222.0)	165.0 (137.0–222.0)	191.0 (167.0–221.0)	160.5 (137.0–205.0)	NS	NS	NS
HDL cholesterol (mg/dl)	48.0 (37.0–56.0)	46.5 (38.0–56.0)	51.5 (40.5–64.5)	39.0 (34.0–48.5)	NS	NS	NS
Triglycerides (mg/dl)	105.5 (66.0–128.0)	80.0 (64.0–126.0)	124.5 (105.5–152.0)	82.5 (79.0–100.0)	NS	NS	NS
LDL cholesterol (mg/dl)	113.0 (78.0–150.0)	101.0 (71.0–150.0)	120.0 (102.0–155.0)	110.5 (84.0–150.0)	NS	NS	NS
Troponin I (ng/ml)	0.7 (0.1–8.5)	1.6 (0.1–9.4)	0.4 (0.1–2.6)	0.3 (0.2–30.0)	NS	NS	NS
Creatinine (mg/dl)	0.9 (0.8–1.3)	1.0 (0.8–1.3)	0.8 (0.8–1.1)	0.9 (0.8–1.7)	NS	NS	NS
Sodium (mmol/l)	140.0 (139.0–141.0)	140.9 (138.1–143.2)	140.3 (139.8–140.7)	139.5 (136.7–141.8)	NS	NS	NS
Potassium (mmol/l)	5.0 (4.0–5.0)	4.3 (3.7–4.6)	4.5 (4.1–4.8)	5.1 (4.1–5.9)	NS	NS	NS
Chloride (mmol/l)	104.0 (103.0–108.0)	105.3 (100.1–109.7)	104.3 (103.6–105.2)	105.6 (103.2–108.6)	NS	NS	NS
Glucose (mg/dl)	110.5 (99.0–154.0)	111.0 (100.0–159.0)	108.0 (99.0–143.0)	125.0 (93.5–169.0)	NS	NS	NS

ACS — acute coronary syndrome; HDL — high density lipoprotein; INR — international normalised ratio; LDL — low density lipoprotein; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration; MCV — mean corpuscular volume; MPV — mean platelet volume; NS — non significant; PCT — plateletcrit; P-LCR — platelet large cell ratio; PDW — platelet distribution width; RDW-CV — red blood cell distribution width coefficient variation; RDW-SD — red blood cell distribution width standard deviation

5–6 changes in measurements using emission spectra or 4-5 changes in measurements using excitation spectra. During all the measurements, the following changes were observed: a shift of the autofluorescence spectra (Fig. 1), and a change of shapes of the spectra (Fig. 2). Only three patients' plasma samples showed entirely differently after deep-freezing when analysing the results of both emission and excitation spectra.

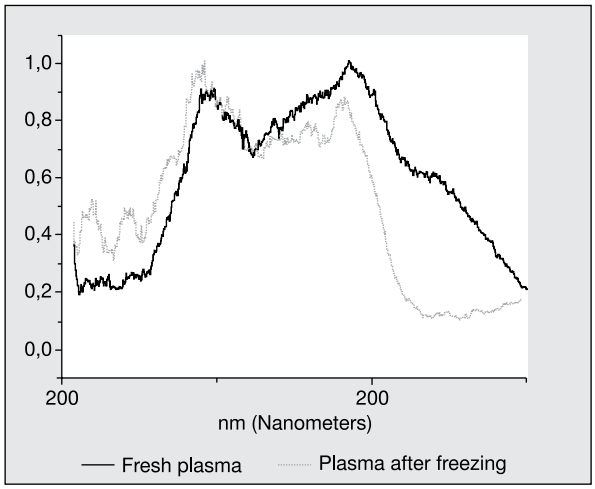

Figure 1. Excitation spectra from fresh plasma and plasma after deep freezing (sample 22; λ = 400 nm)

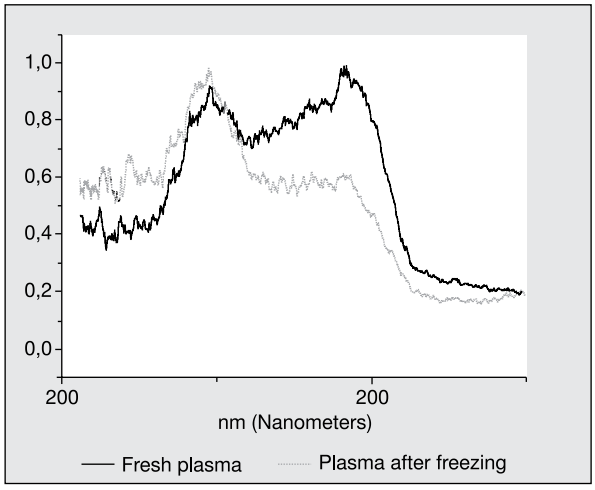

Figure 2. Excitation spectra from fresh plasma and plasma after deep freezing (sample 3; λ = 400 nm)

The second group (subgroups B and B') of plasma samples showed partial changes in the measurements of autofluorescence. By 'partial changes' we mean: 2–4 changes in measurements using emission spectra or 3–4 changes in measurements using excitation spectra. Modifications such as: a shift of the autofluorescence spectra (Fig. 1), and a change in the shape of the spectra (Fig. 2), were observed. Modifications were complex and diversified in different patients (Tab. 3).

The third group (subgroups C and C') of samples was freezing-resistant. Any significant changes comparing plasma samples before and after deep-freezing were found in spectroscopic analysis (Fig. 3). By 'not significant changes' we mean at most one accidental change in the measurements using emission or at most one change in the measurements using excitation spectra. Only two patients' plasma samples were freezing-resistant and presented the same comparable results of both emission and excitation spectra.

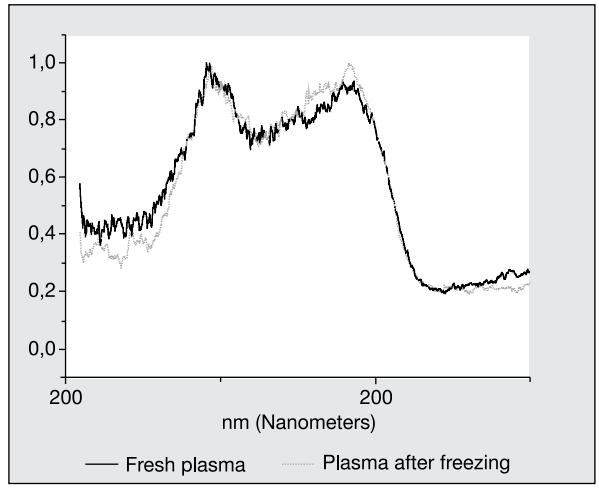

Figure 3. Excitation spectra from fresh plasma and plasma after deep freezing (sample 4; λ = 400 nm)

In addition, we also checked, in a small group of three patients, whether incubation at 37°C influences the plasma spectroscopic properties. Therefore, after thawing, the plasma samples of three randomly selected patients were warmed up to 37°C, which is the normal body temperature. The spectroscopic measurements of the plasma samples were made after 0.5 h in 37°C, then after 1 h and 1.5 h in 37°C. This experiment demonstrated that spectroscopic characterisation of the deep-frozen sample was the same when the plasma was kept at room temperature as it was after incubation at 37 degrees (Fig. 4). Probably, spectroscopic changes which were observed after deep-freezing are connected to irreversible damage caused by the freezing process. This hypothesis requires further investigation.

Discussion

The process of deep-freezing may affect the autofluorescent spectra of some human plasma proteins. It is now well established that proteins undergo cold denaturation at low temperatures as well as at high temperatures [5, 6]. The majority of proteins are sensitive to low temperatures which may cause loss of their native structure/conformation and enhancement of visibility of their non-polar residues. Water molecules tend to form ice-like cages around non-polar molecules. This special structure, known as a clathrate cage, is associated with thermodynamic changes [7, 8]. Compared to heat denaturation, cold denaturation is generally reversible,

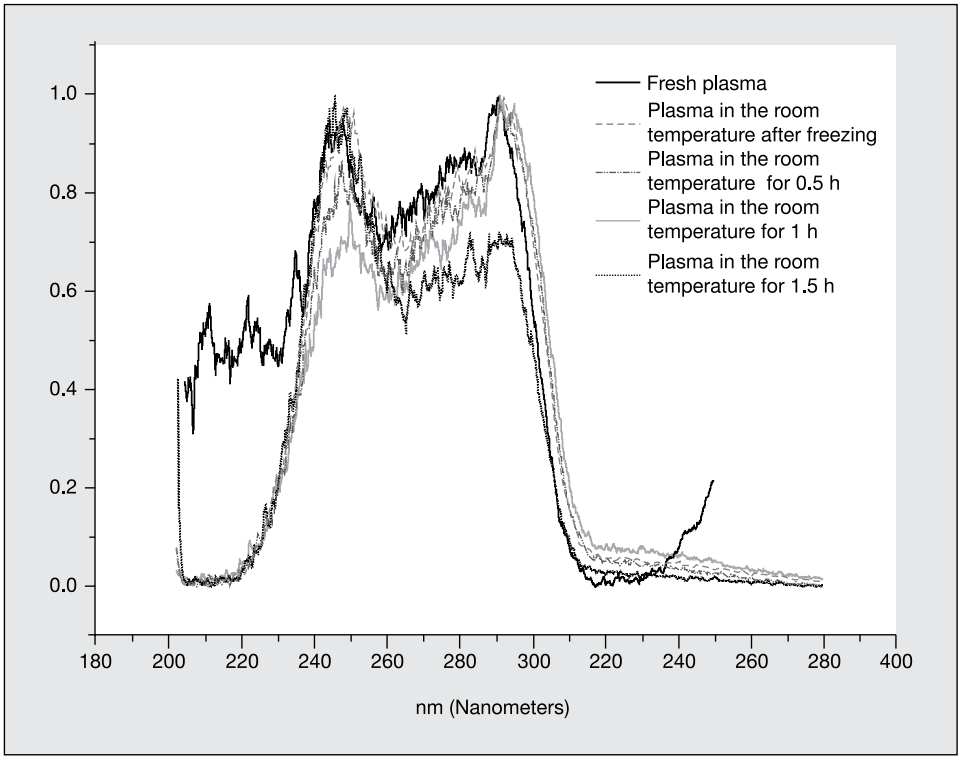

Figure 4. Excitation spectra from fresh plasma and plasma after deep freezing (sample 17; λ = 400 nm)

so proteins can regain their biological functionality. However, some proteins are particularly sensitive to low temperatures, so they can lose their function irreversibly as well.

The formation of ice crystals still remains unexplained. This process occurs upon ice formation via nucleation and crystal growth from supercooled water. There are three stages of this process: chemical diffusion for water molecule transportation, the surface kinetic phase for hydrogen bonds formation, and the heat conduction for heat releasing and diffusing. In fact, the ice crystals formation process is heterogeneous in most cases and may be influenced by many different factors. Until now, the two main theories devised to explain this process can be classified as 'thermodynamic approximation' and 'molecular simulation' [9]. Given the fact that the formation of ice crystals is not fully understood, it can be assumed that it may damage the structure of proteins, which have autofluorescent properties, as well as other proteins, which also contribute to the fluorescence of human plasma.

Probably, during freezing, some proteins which are able to emit autofluorescence are prone to conformational and/or structural changes. The explanation of the specific mechanism responsible for the change of the plasma fluorescent properties during the process of deep-freezing is of significant importance to confirm the reliability of studies using frozen plasma samples. One supposed mechanism involves conformational changes, which may appear during the temperature changes.

Because of the complex nature of the fluorescence phenomenon, all kind of changes concerning the autofluorescent properties of proteins may affect the fluorescence of the whole plasma sample [10]. These changes, which could include reallocation of maximum spectra (Fig. 1) and change of shapes of autofluorescence spectra (Fig. 2), may be caused by environmental modification of the particular protein. Accordingly, the structure or conformational changes in some proteins may change fluorescence, something that puts off fluorescence in certain proteins and puts in fluorescence in other proteins, simultaneously. It may significantly convert shapes of the autofluorescence spectra.

The differences in the spectroscopic properties of plasma among patients may be due to individual variability. Plasma is not only a biological material but also represents a proteome, which is specific for a given patient. It usually contains a vast amount of proteins or their modified forms. There are many factors which may influence plasma proteome such as: age, sex, diet, stress, diseases and many others [11]. Because of these, the analysis of plasma specific protein com-

position without prior separation of the most abundant proteins is very difficult, or even impossible.

It is crucial to continue further investigation in this field. The most significant step is the fractionation of human plasma in order to check the autofluorescent properties of different protein fractions. It is also essential to attempt to recognise the components of appropriate autofluorescence spectra. The most useful available method to separate proteins is two-dimensional electrophoresis [12]. Thereafter, analysis with MALDI-TOF, which is an ionisation technique used in mass spectrometry, will be the next step in research.

Acknowledgements

This study was supported by a research grant from the National Science Centre, Poland (grant no. N N402 497240).

References

1. Teale FWJ, Weber G. Ultraviolet fluorescence of the aromatic amino acids. Biochem J 1957; 65: 467–482.
2. Teale F. The ultraviolet fluorescence of proteins in neutral solution. Biochem J 1960; 76: 381–188.
3. Lakowicz JR. On spectral relaxation in proteins. Photochem Photobiol 2000; 72: 421–437.
4. Lakowicz JR. Principles of Fluorescent Spectroscopy, Dynamics of Solvent and Spectral Relaxation, Springer, New York 2006: 237–244.
5. Dias CL, Ala-Nissila T, Wong-Ekkabut J et al. The hydrophobic effect and its role in cold denaturation. Cryo 2010; 60: 91–99.
6. Dias CL, Ala–Nissila T, Karttunen M et al. Microscopic mechanism for cold denaturationm. Phys Rev Lett 2008; 100: 118101.
7. Head-Gordon T. Is water structure around hydrophobic groups clathrate-like? Proc Natl Acad Sci USA, 1995; 92: 8308–8312.
8. Caldarelli G, De Los Rios P. Cold and warm denaturation of proteins. J Biol Physics 2001; 27: 229–241.
9. He ZZ, Liu J. Modeling ice crystal formation of water in biological system. In: Advanced Topics on Crystal Growth, ed. Sukarno Olavo Ferreira, http://www.intechopen.com/books/advanced-topics-on-crystal-growth/modeling-ice-crystal-formation-of-water-in-biological-system.
10. Lakowicz JR. Principles of Fluorescent Spectroscopy, Protein Fluorescence, Springer, New York 2006: 530–543.
11. Anderson NL, Anderson NG. The Human Plasma Proteome, History, Character, and Diagnostic Prospects. Mol Cell Proteomics, 2002; 1: 845–867.
12. O'Farell PH. High resolution two-dimensional electrophoresis of proteins. The Journal of Biological Chemistry, 1975; 250: 4007–4021.