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Native fluorescence spectroscopy of blood plasma of rats with experimental diabetes: identifying fingerprints of glucose-related metabolic pathways

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Abstract. We present the results of a native fluorescence spectroscopy study of blood plasma of rats with experimental diabetes. It was shown that the fluorescence emission band shape at 320 nm excitation is the most indicative of hyperglycemia in the blood plasma samples. We provide the interpretation of this fact based on the changes in reduced nicotinamide adenine dinucleotide phosphate concentration due to glucose-related metabolic pathways and protein fluorescent cross-linking formation following nonenzymatic glycation. © *2015 Society of Photo-Optical Instrumentation Engineers (SPIE)* [DOI: 10.1117/1.JBO.20.5.051033]

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1 Introduction

Native fluorescence of bioliquids and tissues is extensively used for detection of pathological processes in the human organism. This approach is based on the evaluation of native molecular fluorophores emission, which depends on metabolic and pathological conditions of the organism.[1–9] For instance, numerous works are devoted to cancer detection using tissue and (less frequently) blood serum fluorescence.[5–10]

The pathogenesis of diabetes mellitus, which is one of the most common metabolic diseases in the world,[11] is also studied by fluorescence spectroscopy. Diabetes mellitus is among the diseases with a great impact on health and society, not only due to its high prevalence but also because of its chronic complications and high mortality, so the development of diagnostic methods aimed at its monitoring is of high importance for medicine. The development of diabetes is associated with hyperglycemia, i.e., the increased glucose concentration in blood, which leads to accelerated nonenzymatic glycation of proteins and the formation of advanced glycation end-products (AGEs).[12,13] Some AGEs exhibit strong fluorescence, thus making possible its optical detection—for instance, collagen glycation accompanying diabetes can lead to fluorescent AGEs formation and to skin browning.[14,15] It was also suggested that the glycation level of blood plasma proteins, hemoglobin and albumin can serve as a valuable indicator of diabetes complication.[16]

Previously, the blood plasma of rats was studied by time-domain terahertz spectroscopy. It was demonstrated that the absorption coefficient of blood plasma was significantly reduced depending on the experimental diabetes severity.[17] Here, we studied the fluorescence properties of the same samples of blood plasma of rats with experimental alloxan-induced diabetes to determine fluorescence markers of hyperglycemia. It was shown that the intensity of fluorescence peaks in this case is not indicative of glucose-related metabolic processes, while the spectral band shape of the blood plasma fluorescence emission upon excitation at 320 nm correlates with the biochemically determined glucose concentrations. We provide an interpretation of this marker based on the NAD/reduced nicotinamide adenine dinucleotide phosphate (NADPH) ratio variation and fluorescent protein cross-linking formation accompanying diabetes.

2 Materials and Methods

2.1 *Samples Preparation*

The study was performed on the blood plasma of male Wistar rats. The animals were kept in individual cages on the standard diet of the vivarium with access to food and water *ad libitum*. Experiments were performed in accordance with the ethical principles of the EC Council Directive (86/609/EEC) and Declaration of Helsinki. Experimental diabetes in rats (which led to the development of stable hyperglycemia) was simulated by a single intraperitoneal injection of alloxan (Sigma) dissolved in saline solution at a dose of 170 mg/kg of bodyweight after 18 h of starvation, whereas the control group received only a saline injection.[18,19] Blood plasma samples were collected in test tubes containing heparin from rats 12 days after alloxan administration. Glucose concentration was determined by the enzyme method using GLU kits (Bio-Con). The glucose level was 6.6 ± 0.9 mmol/l in the group of control animals

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($n = 4$) and 23.1 ± 4.8 mmol/l in the group of animals with alloxan-induced diabetes ($n = 9$).

2.2 *Fluorescence Measurements*

Fluorescence spectra were measured using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, Japan-France). Experiments were performed in a 0.5 mL quartz cuvette with a 4 mm optical path. The excitation wavelength was varied in the 280- to 450-nm region with a step of 5 nm. The spectral width of the excitation and emission slits was set to 2 nm. The samples were measured as obtained without any dilution.

3 Results and Discussion

3.1 *Intrinsic Fluorescence of Proteins*

All the investigated samples exhibited strong fluorescence centered at ca. 350 nm upon excitation in the UV spectral region (280 to 310 nm). This peak can be attributed to proteins' intrinsic fluorescence, which is mainly due to tryptophan residues (Trp). Trp fluorescence has been used for decades to study proteins' conformational changes and intermolecular interaction.[20] It is known that Trp fluorescence is influenced by the microenvironment—the position of the maximum of its emission varies from ca. 360 nm for Trp in aqueous solution (high polarity) to 310 nm for Trp residues buried inside hydrophobic regions of protein.[21] Consequently, the changes in protein conformation induce shifts in the Trp fluorescence maximum position and fluorescence quenching of Trp may occur. However, in our experiments, the spectral band shape obtained at 295 nm excitation (when fluorescence is mainly due to Trp) was identical with the maximum located at 350 nm, i.e., we did not observe any proteins' conformational changes in the samples with hyperglycemia from the intrinsic fluorescence data [Fig. 1(a)].

Trp fluorescence of blood plasma was described in Ref. 1. Variation of intrinsic proteins' fluorescence emission band shape was used in the literature to detect pathological processes. In Ref. 22, the authors showed that the variation of protein content in human blood serum results in changes of the fluorescence emission band shape—this fact was interpreted as a consequence of (1) the difference in the maximum positions for albumin and globulins and (2) tumor-associated metabolites binding to proteins. The ratio of peak intensities at 365 and 337 nm ($\lambda_{exc} = 287$ nm) was suggested as an indicator of oncogenic processes. Trp fluorescence variations in the blood serum of human patients were also demonstrated in Ref. 6.

Some works demonstrate the decrease of Trp fluorescence emission due to pathological processes.[22] It is also known that the several times reduction of intrinsic fluorescence intensity accompanies the nonenzymatic glycation of proteins.[23,24] Hence, one could expect the correlation between Trp fluorescence intensity and glucose level in the sample [i.e., group of the sample—a normal one with a glucose concentration of 6.6 ± 0.9 mmol/l or with experimental diabetes (23.1 ± 4.8 mmol/l)]. However, such a correlation was not observed [Fig. 1(b)]. Summarizing, Trp fluorescence did not exhibit any correlations with glucose concentration in the samples.

3.2 *AGE-Related Fluorescence*

Another classical fluorescent fingerprint of diabetes is the AGE-related fluorescence. In blood plasma, nonenzymatic glycation of proteins occurs, resulting in the formation of fluorescent complexes between glucose and amino acid residues.

The kinetics of nonenzymatic glycation was studied for albumin in vitro:[24] it was shown that its incubation with glucose over 20 days results in an almost 10-fold increase of fluorescence intensity at ca. 410 nm ($\lambda_{exc} = 350$ nm). Hence, an increase in AGE-related fluorescence was expected in our experiments for rats with hyperglycemia.

Figure 2(a) demonstrates the fluorescence spectra obtained at 350 nm excitation. We found no correlation between the intensity of the band at 450 nm and increased glucose concentration in the samples [Fig. 2(b)], though some spectral changes were observed. Namely, a ca. 15 nm blueshift was detected for the samples with hyperglycemia.

The possible explanation for the absence of correlation between glucose concentration and AGE-related fluorescence could be the low time of the proteins' incubation with glucose. Blood plasma samples in our experiments were obtained

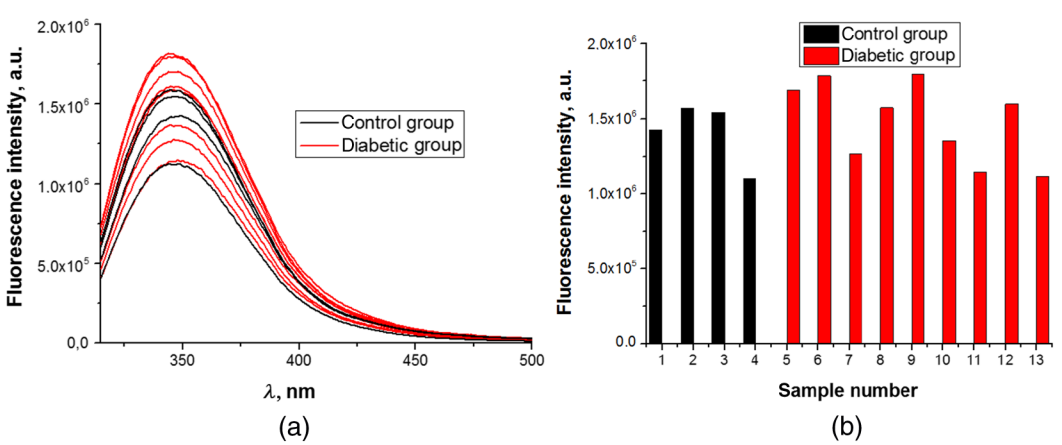

Fig. 1 (a) Fluorescence emission spectra of blood plasma of rats at excitation wavelength $\lambda_{ex} = 295$ nm and (b) dependence of maximum fluorescence intensity of Trp($\lambda_{ex} = 295$ nm) on the sample number. Here and after black and red colors correspond to the control group of rats and rats with hyperglycemia, respectively.

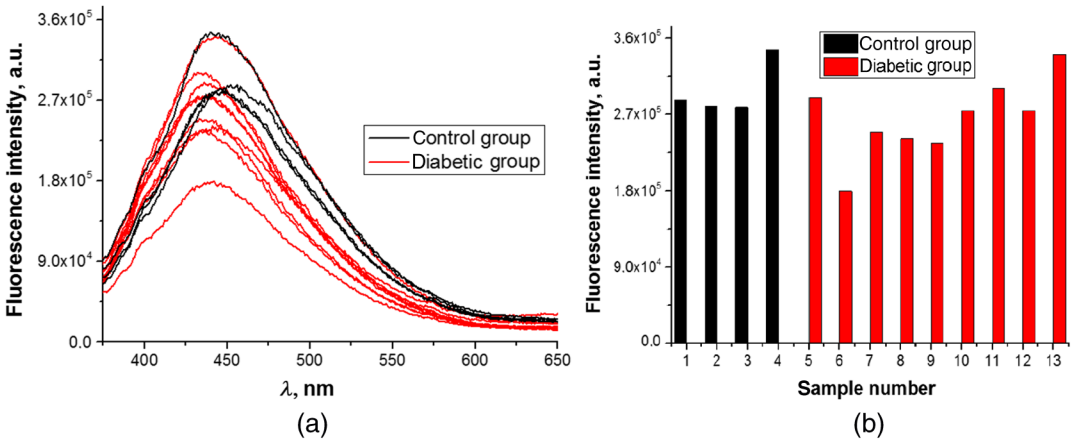

Fig. 2 (a) Fluorescence emission spectra of blood plasma of rats at excitation wavelength $\lambda_{ex} = 350$ nm and (b) the dependence of maximum fluorescence intensity on the sample number at $\lambda_{ex} = 350$ nm.

12 days after alloxan administration, while the observable changes in AGEs fluorescence during albumin glycation appear after 18 days of incubation with glucose.[24] Another reason could be the domination of nonglycated proteins and/or the presence of different metabolites with similar spectral properties in the samples. This hypothesis will be addressed below.

3.3 *320 nm Excitation Provides the Indicator of Hyperglycemia*

The analysis of fluorescence spectra obtained from blood plasma samples revealed that the major differences between the control group of rats and animals with hyperglycemia were observed upon 320 nm excitation. The corresponding spectra are presented in Fig. 3.

It can be seen in Fig. 3 that the spectral band shape is different for the two groups of rats. This difference can be characterized, e.g., by the ratio of intensities at 450 and 390 nm, which varies almost 1.5 times between the control group and rats with hyperglycemia, serving as an indicator of elevated glucose concentration.

In order to investigate the nature of this correlation, we performed a detailed analysis of fluorescence spectra obtained at

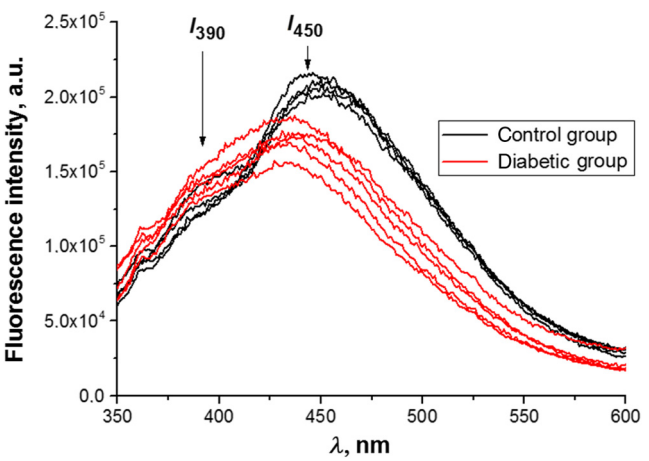

Fig. 3 Fluorescence emission spectra of blood plasma of rats at excitation wavelength $\lambda_{ex} = 320$ nm.

$\lambda_{exc} = 320$ nm. We performed the decomposition of fluorescence spectra, obtained at $\lambda_{exc} = 320$ nm, into Gaussian peaks. The minimum number of peaks needed for adequate spectra decomposition was equal to three, while the decomposition into two peaks was unsatisfactory. The decomposition of fluorescence spectra for the samples from the control group and the group with experimental diabetes is presented in Fig. 4.

Essentially, the positions and FWHMs of all three peaks were close for all the experimental spectra (Table 1), thus confirming the correctness of the decomposition procedure. The first peak P_1 in Fig. 4 can be presumably attributed to Trp fluorescence. The ratio of maximum intensities of the two other peaks P_2 and P_3, I_2/I_3, correlated with the group type of rats (control versus diabetic). This indicator is the analogue of the ratio of peak intensities at the two fixed wavelengths 390 and 450 nm (I_{390}/I_{420}), suggested above. The I_2/I_3 value varied almost two times for the two groups of rats compared to a 1.5 times variation for I_{390}/I_{450}.

The closest analogue of this indicator (I_{450}/I_{390} or I_2/I_3 at $\lambda_{exc} = 320$ nm) that we found in the literature was the indicator for another pathological process (cancer)—the ratio of intensities at 450 and 400 nm in the fluorescence spectra obtained upon excitation at 315 nm.[3,4] The authors studied the fluorescence properties of blood serum for almost 100 patients with different types of cancer and attributed the peak at ca. 450 nm to nicotinamide adenine dinucleotide phosphate [NAD(P)H] fluorescence. The authors suggested that the intensity of this peak could serve as an integral indicator of energy metabolism and that NAD(P)H concentration (and, consequently, fluorescence) decreases due to oxidative stress and damage of cells (e.g., red blood cells) by reactive oxygen species following pathological processes. We note that the concentration of AGEs strongly correlates with oxidative stress (advanced oxidation protein products) in patients with diabetes mellitus.[25] The authors[3] also provided biochemical data supporting the hypothesis of reduced energy metabolism in cancer bearing patients, leading to the changes in NAD(P)H fluorescence.

It is known that NAD(P)H is involved in a variety of metabolic processes, including glucose-related metabolic pathways.[26–28] Hence, we analyzed the possibility that NAD(P)H concentration changes could be the origin of the observed correlation between the spectral characteristics and the type of the sample in our experiments.

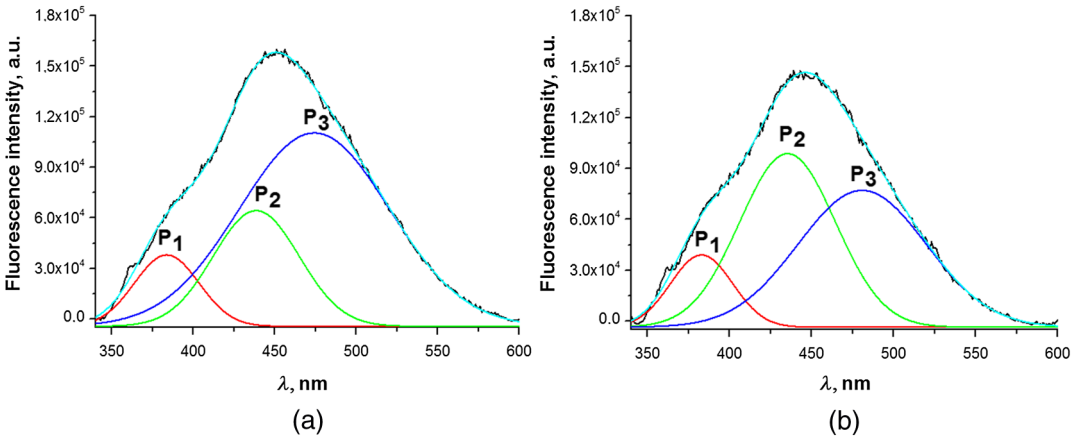

Fig. 4 Fluorescence emission spectra of blood plasma of rats for control group (a) and group with hyperglycemia (b) obtained upon $\lambda_{exc} = 320$ nm. Black lines indicate experimental emission spectra of blood plasma and colored lines indicate their fitting by a sum of three Gaussians.

Table 1 Parameters extracted from fluorescence spectra measured upon 320 nm excitation. λ_i and W_i are the positions of maximum and FWHM of P_i; I_2/I_3 and I_{390}/I_{450} are the ratios of maximum intensities of P_2 and P_3 and at two fixed wavelengths (390 and 450 nm), respectively.

Group type	λ_1, nm	λ_2, nm	λ_3, nm	W_1, nm	W_2, nm	W_3, nm	I_2/I_3	I_{390}/I_{450}
Control	383.7 ± 1.7	439 ± 0.5	474 ± 1.7	37.2 ± 2.2	52.5 ± 4.1	90.7 ± 1.2	0.62 ± 0.04	0.44 ± 0.05
Diabetic	381.1 ± 2.8	438 ± 4	473 ± 3.8	36.5 ± 1.5	61.6 ± 3.1	86.6 ± 4.7	1.3 ± 0.1	0.68 ± 0.09

3.4 *NAD(P)H Variations Due to Glucose-Related Pathways*

Nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and NADPH are strongly involved in energy metabolism in organisms, and a change of cytosolic free NAD/NADH could serve as an indicator of the physiological or pathological states, including the pathogenesis of diabetes.[26]

For instance, vasodilation and increased blood flow, which are among the earliest vascular changes associated with diabetes,[27] are closely related to an increase in $NADH/NAD^+$ concentrations due to impaired oxidation of NADH to NAD. In Ref. 27, the authors demonstrated several parallels between functional abnormalities associated with an increased $NADH/NAD^+$ level in diabetic tissues and in hypoxic or ischemic myocardium. In Ref. 28, the authors showed that NADH is the sensor of blood flow need in brain, muscle, and other tissues and that accumulation of electrons in NADH signals blood-flow need and regulates the flow in resting and working tissues. Observations of diabetic animals in Ref. 28 demonstrated that the accumulation of electrons in NADH might augment blood flow.

These facts suggest that NAD(P)H-related fluorescence could be different for the blood plasma of rats from the control group and rats with experimental diabetes. Fluorescence intensity in the 450- to 500-nm region at 350 nm excitation [typical parameters used for NAD(P)H excitation[29]] did not exhibit a correlation with the type of the sample in our experiments (Fig. 2). However, fluorescence emission bands of NAD(P)H and AGEs strongly overlap[23,24,30] and the overall fluorescence spectra could be the composition of several components, thus making a direct comparison between NAD(P)H concentration and fluorescence intensity incorrect. Indeed, it can be seen in Fig. 4 that the fluorescence spectra obtained at 320 nm excitation represent a composition of three bands with the maxima centered at ca. 383, 438, and 474 nm, and in the samples with hyperglycemia, the ratio of the intensities of the second and the third peaks is increased almost twofold. This led us to the necessity to analyze the origin of I_2/I_3 correlation with elevated glucose concentration in the samples.

3.5 *Excitation-Emission Matrix Fluorescence—NAD(P)H and Glycated Proteins*

We analyzed the excitation-emission matrix (EEM) fluorescence patterns of the blood plasma samples to obtain the indicator of glucose-specific processes by studying the cross-sections of the EEMs (Fig. 5). We found that only the cross-sections in the region marked with the red frame in Fig. 5 are indicative of hyperglycemia. This region represents a valley located between two high-intensity peaks Π_1 and Π_2, the first one due to Trp emission (280/350 nm) and the second one (350/450 nm) is a composition of several overlapping fluorescence bands. The cross-sections that belong to this region (including the fluorescence spectra obtained at 320 nm excitation) characterize the shape of the second peak (Π_2). Indeed, the decomposition of fluorescence spectra presented in Fig. 4 demonstrates that P_2 is the sum of at least three components.

In vitro incubation of albumin with glucose demonstrated that a new fluorescence band, which is due to fluorescent cross-linking formation,[30,31] appears in the 400- to 430-nm area of the spectrum upon excitation in the 320- to 350-nm region.

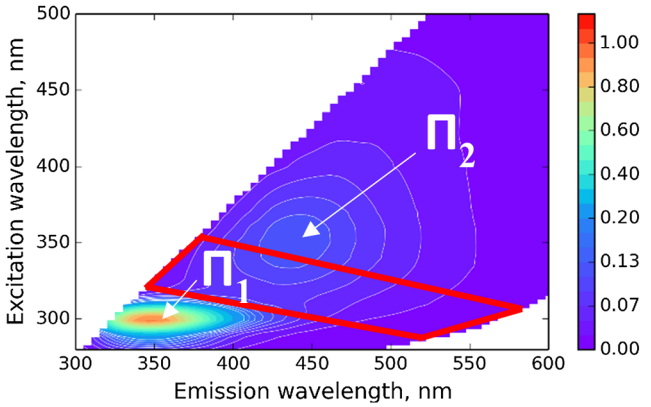

Fig. 5 Excitation-emission matrix fluorescence of blood plasma of rats.

The parameters of this band are close for different proteins (e.g., for albumin and collagen[32]), and one of the major glycation products that causes this fluorescence is supposed to be pentosidine.[33] Fluorescence spectra of NAD(P)H and AGEs overlap strongly; however, NAD(P)H fluorescence can be considered to be more redshifted (462 to 470 nm upon 340 nm excitation[1]). Hence, we suppose that the blueshifted peak P_2 (with its maximum at ca. 440 nm) in Fig. 4 is due to fluorescent cross-linking formation in blood plasma proteins, while the more redshifted peak P_3 (with its maximum at ca. 470 nm) is due to NAD(P)H emission. In this case, the indicator of hyperglycemia observed in this work reveals the changes of both glycation products and NAD(P)H concentrations in blood plasma samples—the ratio of AGEs to NAD(P)H fluorescence intensities (I_2/I_3) is almost increased by twofold for the samples with hyperglycemia.

4 Conclusion

Native fluorescence spectroscopy of the blood plasma of rats with experimental diabetes was studied in this work to identify the fluorescence fingerprints of glucose-related metabolic pathways. It was observed that the fluorescence emission band shape at 320 nm excitation was indicative of hyperglycemia. The fluorescence spectra obtained at 320 nm excitation represent a composition of three bands with their maxima centered at ca. 383, 438, and 474 nm (Fig. 4), and in the samples with hyperglycemia, the ratio of the intensities of the second and the third peaks is increased almost twofold. Based on the literature data, we suppose that the second peak is due to fluorescent cross-linking formation, while the third peak is due to NADPH fluorescence, and the concentrations of these components vary due to glucose-related metabolic pathways.

Acknowledgments

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Biographies of the authors are not available.