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Fluorescence detection of biological objects with ultraviolet and visible light-emitting diodes

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Recent progress in fabrication of semiconductor light emitting diodes (LEDs) allows these devices to be used for excitation of fluorescence of aromatic amino acids and other biofluorophores. In our work, a deep-UV UVTOP^{TM} LED (280 nm) developed by Sensor Electronic Technology, Inc., was used for fluorescence characterisation of natural protein fluorophores in enzyme glucose oxidase (GOx) and in *Bacillus subtilus* dry spores (*B. subtilus*). A longer-wavelength Nichia LED (375 nm) and high-power Luxeon^{TM} LED (450 nm) were used for fluorescence detection of enzyme cofactors. Combined spectral and fluorescence lifetime measurements using selective LED excitation enabled us to recognise the impact of specific autofluorophores in complex biological systems. Inexpensive LED-based fluorescence detectors can be used in designing biosensors and detect-to-warn systems.

Keywords: semiconductor light emitting diodes (LEDs), UV LEDs, fluorescence lifetime, frequency -domain measurements, glucose oxidase, Bacillus subtilus spores.

1. Introduction

Modern sensors are aimed to be used in simple, fast and small electronic devices for our daily routine. In many cases optically based methods of detection of organic materials are faster than other physical and chemical methods. The optical part of a sensor can be inexpensive and compact part of a biochemical device. Recent progress in the production of light-emitting diodes (LEDs) resulted in the development of compact, inexpensive, efficient, stable, durable, longevous and lightweight sources of light [1, 2]. LEDs are available within a variety of emission wavelengths that can be tailored for fluorescence excitation in particular objects. Another advantage is small optical noise, which allows weak fluorescence signals to be measured with the use of LED-based instruments. In addition, LEDs can be operated in a pulsed

and high-frequency harmonical regime for time-resolved and frequency-domain measurements of fluorescence decay time, respectively.

In our previous work [3] we reported on the possibility of applying deep-ultraviolet (deep-UV) LEDs for fluorescence excitation in natural biofluorophores. The studies performed confirmed the applicability of newly developed deep-UV LEDs for fluorescence excitation and fluorescence lifetime measurements in basic protein autofluorophores, tyrosine and tryptophan. LEDs with longer wavelengths are applicable to detection of enzyme cofactors, NADH and riboflavin. Fluorescence lifetimes of the fluorophores ranging from sub-nanoseconds were successfully detected.

In the present work, the applicability of LED excitation for detection of autofluorescence of more complex biological systems such as glucose oxidase (GOx) and *Bacillus subtilus* spores (*B. subtilus*) is shown. The applied selective excitation was shown to be suitable for spectral and fluorescence-lifetime identification of biofluorophores in complex systems. Therefore, this technique can be used in designing of biorecognition devices.

2. Fluorescence detection of biological objects

To demonstrate the availability of LEDs for optical sensing of biological compounds, we performed spectral measurements of fluorescence in enzyme GOx, which is widely used in sensors of glucose, and *B. subtilus* spores, which are the imitator of hazardous biological agent *B. anthracis*. A deep-UV LED with the emission wavelength of 280 nm (Sensor Electronic Technology, Inc.) was utilized for excitation of protein fluorophores (tyrosine and tryptophan residuals) in GOx and *B. subtilus*. LEDs with the emission wavelengths of 375 nm (Nichia) and 450 nm (Philips Lumileds Lighting) were used for excitation of flavins (flavin adenine dinucleotide – FAD) and nicotinamide adenine dinucleotide (NADH).

The emission spectra were measured by a low-stray-light double monochromator (JY HRD1) and a photomultiplier (Hamamatsu R1463P) operating in the photon--counting mode. The experimental set-up used for frequency-domain fluorescence decay measurements is described in [3].

The GOx enzyme is a homodimeric glycoprotein with one noncovalently bound FAD cofactor per monomer; it has 583 amino acid residues and consists of two separate structural domains [4]. The cofactor FAD is located in the first domain and is surrounded by 31 residues, of which four aromatic residues of Tyr-515, Trp-426, Tyr-68, and Trp-111 are in close contact with the cofactor [4]. The GOx absorption spectrum consists of two bands peaking at 380 and 450 nm [5] and corresponding to π-π^* transitions along the three cycles of the isoalloxazine ring in FAD. These bands are characteristic of the oxidized form of flavin groups [6]. In our experiments, we used GOx from *Aspergillus niger* aqueous solution in the sodium acetate buffer at pH 6 with a concentration of 7.25 μM.

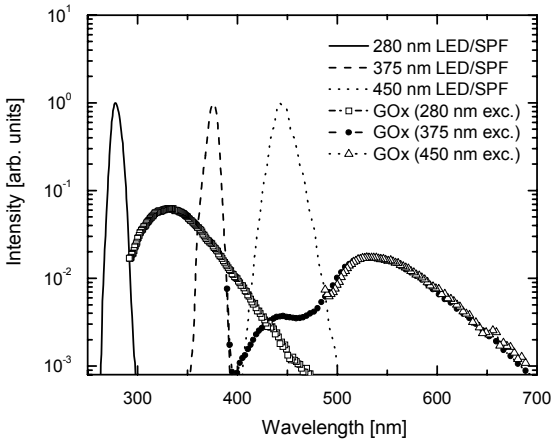

Fig. 1. Fluorescence spectra of GOx under excitation of LEDs with different emission wavelengths (open squares, solid circles and open triangles). Spectra of the excitation emission filtered by short-pass filters (SPF) for 280 nm (solid line), 375 nm (dashed line) and 450 nm (dotted line) LEDs.

Figure 1 shows fluorescence spectra of GOx obtained under excitation of 280, 375 and 450 nm LEDs. For different excitation wavelengths, the spectra are dissimilar and peak in different spectral ranges. The 280 nm LED selectively excites aromatic amino acid residues in GOx, with the fluorescence spectrum containing one band at 330 nm [7]. The fluorescence spectrum for 375 nm excitation consists of two bands peaked at 440 and 530 nm. The longer-wavelength band coincides with that obtained under 450 nm excitation. The band at 440 nm can be attributed to fluorescence of semiquinoid form of flavins. The band at 530 nm corresponds to FAD fluorescence.

Figures 2**a** and 2**b** depict frequency-domain fluorescence decay measurements for GOx enzyme obtained for excitation by the 280 nm and 375 nm LEDs, respectively. Figure 2**c** shows the difference of the fluorescence phase shifts for 280 and 375 nm LED excitation as a function of frequency. The frequency dependences of the phase shift and modulation depth were fitted with a two-exponential model: the fluorescence decay times were $\tau = 4.8 \pm 0.2$ ns and $\tau = 0.92 \pm 0.1$ ns (Fig. 2**a**), $\tau = 5.52 \pm 0.2$ ns and $\tau = 0.65 \pm 0.1$ ns (Fig. 2**b**). The lifetime of about 5 ns corresponds to FAD and is a typical value for flavins [8]. The shorter lifetime can be due to quenching by surrounding groups [4]. The frequency dependence of the phase shift difference with a minimum at 45 MHz can be treated as a "fingerprint" of given compound. We suggest that such a fingerprint can be used for the identification of a biological object.

UV LEDs (280 and 375 nm) were also used for fluorescence identification of *B. subtilus* spores. *B. subtilus* spores are the model imitator of hazardous biological agent *B. anthracis*. Figure 3 shows fluorescence spectra of *B. subtilus* (Hoechst Marion Roussel, Romainville, France). The spectra are seen to be different for each excitation wavelength. Under the 280 nm excitation, the spectrum contains two bands: a dominating band at 329 nm, which can be attributed to tryptophan and tyrosine

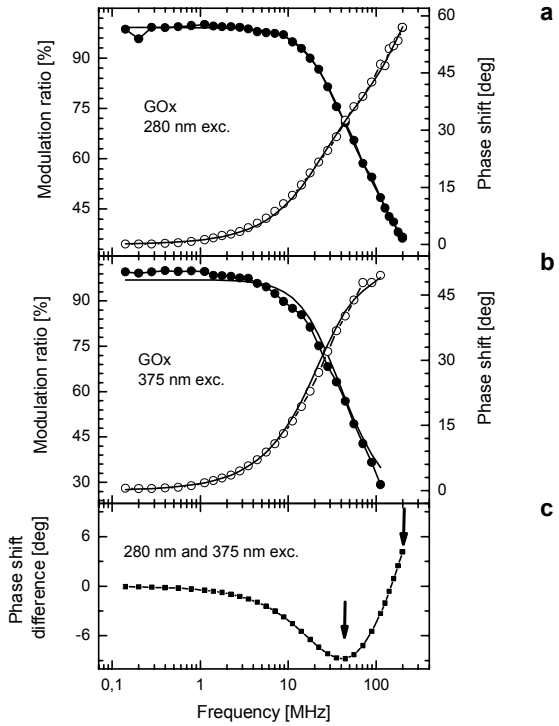

Fig. 2. Phase shift (empty circles) and modulation (full circles) as a function of frequency for GOx under 280 nm LED (**a**) and 375 nm LED (**b**) excitation. The lines correspond to two-exponent fluorescence decay fit with the lifetimes of $\tau = 4.8 \pm 0.2$ ns and $\tau = 0.92 \pm 0.1$ ns (**a**), $\tau = 5.52 \pm 0.2$ ns and $\tau = 0.65 \pm 0.1$ ns (**b**). Frequency dependence of fluorescence phase shift difference between 280 nm and 375 nm LEDs excitation (**c**).

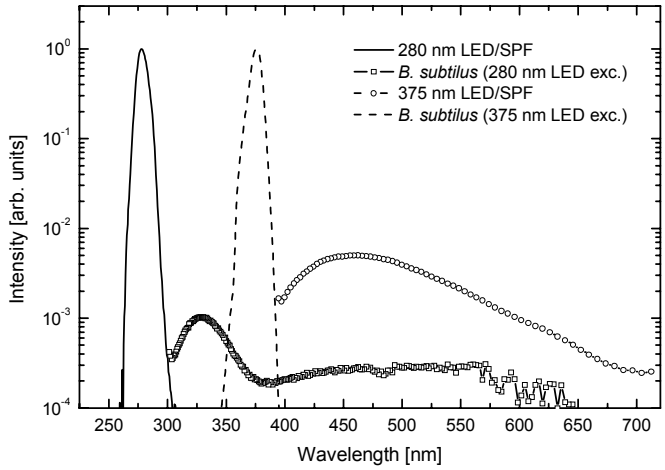

Fig. 3. Fluorescence spectra of *B. subtilus* spores (squares and circles) under 280 nm LED excitation (solid line) and 375 nm LED excitation (dashed line), respectively.

residuals [7], and a weak broad band consisting of two overlapping bands, which can be attributed to fluorescence of NADH and flavins. Under 375 nm excitation, the spectrum exhibits a broad band peaked at 458 nm, which can be attributed to fluorescence of NADH in inhomogeneous environment [8].

The same two excitation wavelengths were used for fluorescence decay measurements in *B. subtilus* by the frequency-domain method. The experimental data for *B. subtilus* shown in Fig. 4 were fitted with a two-exponential model. The extracted fluorescence decay times are $\tau = 0.43 \pm 0.1$ ns and $\tau = 2.91 \pm 0.1$ ns for deep-UV 280 nm excitation. For 375 nm excitation, the fitting procedure revealed two fluorescence lifetimes of $\tau = 1.6 \pm 0.1$ ns and $\tau = 7.1 \pm 0.1$ ns. The main impact in fluorescence identification of *B. subtilus* spores is due to three natural biofluorophores (tryptophan residuals, NADH and flavins), the decay time of which is sensitive to their local environment. Fluorescence lifetime of NADH can increase from 0.4 to 5 ns by bounding to proteins; tryptophan can show fluorescence lifetimes from 1 to 6 ns due to binding of ligands, protein-protein association or quenching by surrounding groups [8].

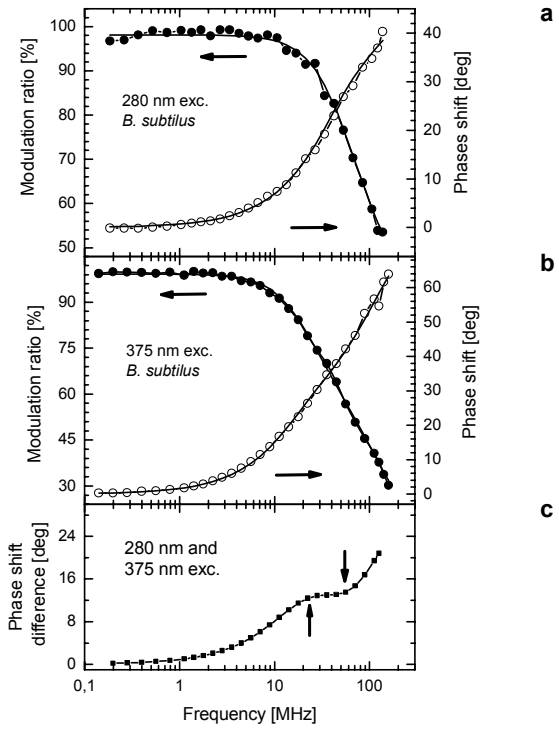

Fig. 4. Phase shift (empty circles) and modulation (full circles) as a function of frequency for *B. subtilus* spores under 280 nm LED (**a**) and 375 nm LED (**b**) excitation. The lines correspond to two-exponent fluorescence decay fit with the lifetimes of $\tau = 0.43 \pm 0.1$ ns and $\tau = 2.91 \pm 0.1$ ns (**a**), $\tau = 1.6 \pm 0.1$ ns and $\tau = 7.1 \pm 0.1$ ns (**b**). Frequency dependence of fluorescence phase shift difference between 280 nm and 375 nm LEDs excitation (**c**).

The specific frequency dependence of fluorescence phase shift difference for *B. subtilus* spores (see Fig. 4**c**) shows two characteristic two points at 24 and at 55 MHz (indicated by arrows in Fig. 4**c**). Again, such characteristic frequency can be reliably measured and provides additional information for identification of a biological object.

3. Conclusions

The LEDs' emission wavelengths, optical output and modulation depth in a wide frequency range were shown to be suitable for measurements of fluorescence spectra and of fluorescence decay in the frequency domain of the complex biological compounds GOx enzyme and *B. subtilus* spores. Selective excitation combined with spectral and frequency-domain measurements allowed for identification of different autofluorophores: proteins, NADH and flavins in complex biological systems. In particular, fluorescence of *B. subtilus* exhibits specific spectral and decay features that can serve as a specific fingerprint in fluorescence sensing of airborne biological pathogens. We suggest that fluorescence identification under LED-excitation became a viable technique for devising compact and inexpensive sensors for biology and biochemistry.

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