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Deconvolution of fluorescence spectra: Contribution to the structural analysis of complex molecules

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Abstract

Fluorescence spectroscopy is a sensitive analytical tool in the studies of both simple and complex molecular structures. In complex molecules, however, determining the number and position of components may give a specific insight into the structure, complementary to the other analytical techniques. We applied log–normal model to analyze fluorescence of simple monofluorophore molecule. In order to analyze spectra where both fluorophores and Raman emission bands were present, we developed a method obtained by combination of the symmetric, Gaussian, for Raman and asymmetric, log–normal model, for fluorescence, applicable to the molecules of different complexity. Technically, for each sample we varied excitation wavelength with 5 nm step and recorded the corresponding emission spectra. They were subsequently used for component analysis. Position of each component was plotted against the excitation wavelength. Applying this approach we could identify minimal number of components having stable positions, while their approximate probability density (APD) in a spectral series was correlated with the probable number of fluorophores in the molecule. The method was tested on molecules containing different number of fluorophores: monomers involved in the synthesis of plant polymer lignin—coniferyl alcohol (one fluorophore), ferulic acid (two fluorophores) and on lignin model compound produced from these monomers (many fluorophores). All investigated species belong to benzene-substituted class of compounds, and it is reasonable to assume that they have similar fluorescence band contour. We also report the results of environmental scanning electron microscopy (ESEM) studies showing multilayered dehydrogenative polymer (DHP) structure, in order to show complexity of the polymer. Our results present complementarity of these two approaches in the structural studies of the lignin model compound.

Keywords: Environmental scanning electron microscopy; Fluorophores; Gaussian model; Lignin model compound; Log–normal model

1. Introduction

Fluorescence spectroscopy is a sensitive analytical tool in the studies of both simple and complex molecules. In complex molecules, however, determining the number and position of fluorophores is difficult task, but it may give a specific insight into the structure, complementary to the other analytical techniques. The analysis of fluorescent spectra of polymeric molecules is especially complex, since they may contain different fluorophores or fluorophores in various microenvironments.

Lignin is the second most abundant polymer on Earth. In plants, it is intertwined and cross-linked with other macromolecules in the cell walls [1]. Fluorescence is an intrinsic property of lignin [2]. Fluorescence spectroscopy was used in the studies of lignin constituents in waters and soils, as well as in photochemistry of wood fibers and paper [3–5]. The structural complexity of lignin makes its fluorescence spectra difficult to interpret.

In our previous work we applied Gaussian model for deconvolution of fluorescence spectra of a lignin model compound [6]. Siano and Metzler [7] introduced the log–normal distribution, as an asymmetric model for the absorption spectra of the hydroxypyridine derivatives in solution. Burstein and Emelyanenko [8] used its mirror-symmetric form as a model for emission spectra

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of different classes of the organic fluorophores. Burstein et al. [9] applied this model to deconvolute spectra of proteins containing tryptophan fluorophore in different microenvironments. Asymmetry is the consequence of the fact that the vibrational force constants of the ground and excited states are not the same.

In this work we investigated the log–normal method, as well as a method obtained by combination of asymmetric (log–normal) and symmetric (Gaussian) model, for analysis of fluorescence spectra of biologically important molecules. The method is tested on molecules of different complexity, in order to confirm its reliability for complex spectra. We analyzed fluorescence spectra of a simple molecule containing one fluorophore, coniferyl alcohol (which is a lignin precursor), a molecule containing two fluorophores, ferulic acid (which is a constituent of natural lignin) and a complex multifluorophore molecule obtained by polymerization of coniferyl alcohol, dehydrogenative polymer (DHP). All investigated species belong to benzene-substituted class of compounds, and it is reasonable to assume that they have similar fluorescence band contour.

Since the Raman bands accompany fluorescence, in this work we report a new model obtained by combination of the symmetric Gaussian, for Raman, and asymmetric, log–normal model, for fluorescence. This combination was applied in case of ferulic acid and DHP, while fluorescence of coniferyl alcohol was fitted with log–normal model only, since no Raman band was detected.

We also report the results of environmental scanning electron microscopy (ESEM) studies showing multilayered DHP structure, in order to show complexity of the polymer. Our results present complementarity of these two approaches in the structural studies of the lignin model compound.

2. Materials and methods

2.1. Synthesis of the dehydrogenative polymer

Lignin model dehydrogenative polymer was synthesized according to the procedures of Freudenberg [10] and Wayman and Obiaga [11], as was reported in the publications of Radotić et al. [12,13]. DHP was synthesized from coniferyl alcohol, using horseradish peroxidase as an enzymatic catalyst. The reaction mixture contained 5×10^{-3} M coniferyl alcohol, 5×10^{-3} M H_2O_2 and 2.5×10^{-8} M horseradish peroxidase (all from Fluka Chemical Corp., New York) in 50 mM phosphate buffer. The reaction mixture was prepared by simultaneous addition of H_2O_2 and coniferyl alcohol solutions to peroxidase solution. After mixing, the solution was shaken constantly for 48 h. Polymerization occurs in solution phase, at a temperature of 25 °C. Reactions took 48 h to complete. The precipitate was washed twice in deionized water and evaporated in a vacuum at 5 °C.

Ferulic acid was obtained from Fluka Chemical Corp.

1,4-Dioxane (Sigma–Aldrich Corp., St Louis) was distilled before use. In all experiments deionized water was used.

2.2. Steady-state fluorescence spectroscopy

Fluorescence spectra were collected using a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Paris, France) equipped with a 450 W xenon lamp and a photomultiplier tube. Coniferyl alcohol, ferulic acid and lignin model compound were dissolved in dioxane/water (9:1, v/v), in a 1-cm optical path length quartz cuvette. The concentration of all the studied samples was 0.5 mg mL^{-1}. The slits on the excitation and emission beams were fixed at 4 and 2 nm, respectively. The spectra were corrected for the dark counts. In each measurement an optimal number of scans was averaged. The emission spectrum of the solvent (dioxane/water) was subtracted. All measurements were performed at controlled temperature of 25 °C by means of a Peltier element.

For each compound a series of emission spectra was collected, by excitation at different wavelengths, starting from 290 nm for coniferyl alcohol, 340 nm for ferulic acid and 360 nm for DHP, with 5 nm step.

Nonlinear fitting of all fluorescence spectra was performed using the Nelder–Mead simplex algorithm implemented in Matlab 6.5. Deconvolution into asymmetric (log–normal) and symmetric (Gaussian) components was applied when both fluorophores and Raman emission bands were present in the spectra.

Samples for ESEM imaging were prepared from water suspension of the lignin DHP polymer. The water suspension of lignin was prepared by sonification, using an ultrasound bath for 60 s. A drop of suspension was placed on a highly oriented pyrolytic graphite (HOPG) substrate (Advanced Ceramics Corporation, Cleveland, Ohio, USA). The substrate had been previously glued with double-sided carbon tape to the ESEM sample holder. The substrate, with deposited films, was then placed in the chamber of a FeiCo-Phillips-Electroscan FEG XL-30-ESEM field emission gun ESEM. Previously, it has been shown that field emission gun SEM and ESEM could be successfully used for investigating the surface morphology of self-assembled and Langmuir Blodgett films [14] without applying additional sample treatment, such as coating, thus avoiding the introduction of artifacts. All imaging was performed in wet mode, with 1 Torr of H_2O as an imaging gas. The imaging signal was gaseous secondary electron (GSE) signal. For the purpose of this study, we used the wide field GSE detector, without an external pressure-limiting aperture.

3. Results and discussion

A typical result of component log–normal deconvolution of coniferyl alcohol emission spectrum obtained by excitation at 290 nm, is shown in Fig. 1. The spectrum was fitted with one log–normal component (Fig. 1, left panel), additional band did not improve accuracy. No indication for Raman band was found. Fig. 1, right panel, shows position of the component of coniferyl alcohol emission spectra, for all excitation wavelengths. Component position exhibited relative stability. It is evident that emission spectrum of coniferyl alcohol consists of only one component at 347 nm (fluorophore).