Conference materials
UDC 544.536
DOI: https://doi.org/10.18721/JPM.183.257

Fluorescent micropatterning of an albumin film with high intensity femtosecond laser pulses

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Abstract. In this study, we performed laser ablation of the solid albumin film, using near-infrared high intensity femtosecond laser pulses. As a result, microstructures with multicolor and excitation-dependent visible fluorescence were produced. We studied fluorescence characteristics of laser-processed protein and characterized its chemical modifications.

Keywords: proteins, femtosecond laser pulses, fluorescence, fluorescence patterning, laser microstructuring

Citation: Astafiev A.A., Shakhov A.M., Syrchina M.S., Nadtochenko V.A., Fluorescent micropatterning of an albumin film with high intensity femtosecond laser pulses, St. Petersburg State Polytechnical University Journal. Physics and Mathematics. 18 (3.2) (2025) 284–288. DOI: https://doi.org/10.18721/JPM.183.257

Материалы конференции
УДК 544.536
DOI: https://doi.org/10.18721/JPM.183.257

Создание флуоресцентных микроструктур в дегидратированной пленке альбумина при помощи высокоинтенсивных фемтосекундных лазерных импульсов

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Аннотация. В настоящей работе продемонстрировано микроструктурирование альбуминовой пленки при помощи остросфокусированного фемтосекундного лазерного излучения ближнего ИК-диапазона. Результатом микроструктурирования стало образование продуктов с многоцветной и зависящей от длины волны флуоресценции. В работе приведены оптические и физико-химические характеристики продуктов лазерного воздействия.

Ключевые слова: белки, фемтосекундные лазерные импульсы, флуоресценция, лазерное микроструктурирование

Ссылка при цитировании: Астафьев А.А., Шахов А.М., Сырчина М.С., Надточенко В.А. Создание флуоресцентных микроструктур в дегидратированной пленке альбумина при помощи высокоинтенсивных фемтосекундных лазерных импульсов // Научно-технические ведомости СПбГПУ. Физико-математические науки. 2025. Т. 18. № 3.2. С. 284–288. DOI: https://doi.org/10.18721/JPM.183.257

Introduction

Laser writing of fluorescent micropatterns in organic matrices holds promise for many applications, including counterfeiting, data storage, bioengineering, optical devices and sensors [1–4]. Patterning with ultrashort laser pulses minimizes thermal damage to the material and enables spatially localized patterning and even direct writing of 3-dimensional structures [5]. Biopolymers, e.g., cellulose, are attractive as a target material in fluorescent writing due to their superior biocompatibility [6]. Proteins, another class of natural biopolymers with highly versatile properties, are a promising material with yet unexplored potential for laser writing. Proteins exhibit weak intrinsic visible fluorescence, which can be enhanced by aggregation or by photooxidation of aromatic residues [7–8]. Both mechanisms enable manipulation of fluorescence using laser irradiation. Previously we demonstrated that a model protein – bovine serum albumin (BSA). A is rendered highly fluorescent upon irradiation with femtosecond laser pulses in a solution [9]. Here were examined if femtosecond laser modification of the solid BSA enables direct wiring of fluorescent micropatterns in a protein film and analyzed fluorescent properties of laser-processed protein and their relation to the processing laser parameters. Chemical modifications of the BSA upon laser irradiation were probed using Raman and infrared spectroscopy and the mechanism of laser modification analyzed.

Materials and methods

Laser processing of the protein films. Protein films were obtained by drying a drop of the BSA aqueous solution on a silica glass surface at a room temperature. Femtosecond laser pulses generated by a TETA laser system (Avesta Project, central wavelength 1033 nm, pulse duration 260 fs, repetition rate 10 kHz, pulse energy up to 1 μJ) were coupled to an optical microscope (Olympus IX71) and focused on the surface of the BSA film with an objective lens (Olympus, NA = 0.75). The beam waist diameter at the focus was estimated as 1.7 μm. With corrections for losses and the objective lens transmission the estimated laser fluence J was related to the input laser power P as $J[J/cm^2] = 0.9 \cdot P$ [mW]. Laser fluence in the focal area reached values about 10 J/cm^2, which caused ablation of the protein films. The BSA film was processed with laser pulses in a square area of 30–100 μm width by moving the film relative to the objective lens focal point using a programmable piezoelectric stage (NT-MDT, scanning speed 50 μm/s). (Fig. 1, *a*). Several squares were created in a film with a laser power ranging from 2 to 10 mW and one scanning cycle. Another series of laser-processed squares was created with a laser power fixed at 5 mW and the number of successive cycles varying from 1 to 32.

Samples characterization. Fluorescent images and fluorescence emission spectra of the protein film were obtained with a scanning confocal microscope (LSM 980, Zeiss Microscopy) using

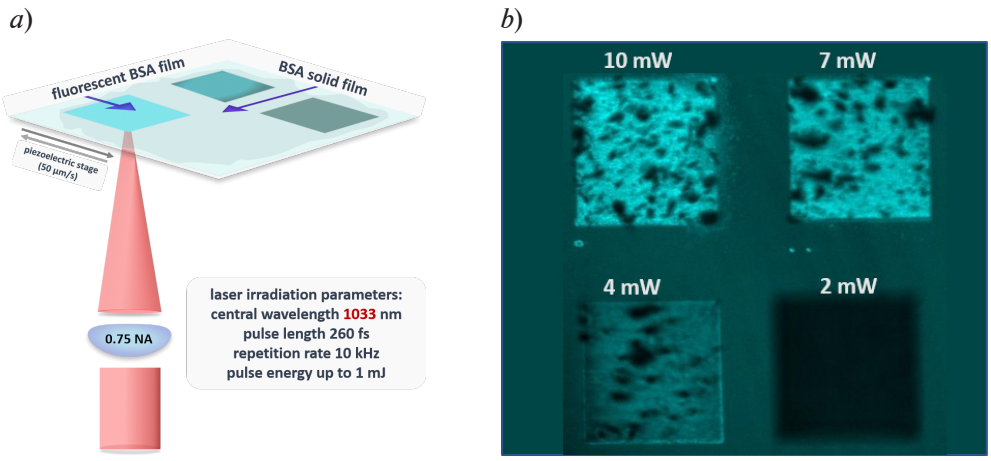

Fig. 1. Experimental setup (*a*); fluorescent confocal images of micropatterns (*b*), written in the BSA film by femtosecond laser irradiation with laser powers of 2 mW, 4 mW, 7 mW, 10 mW

fluorescence excitation at 405, 488 and 561 nm. Fluorescence emission spectra were averaged over processed squares. Raman spectra of the BSA were registered locally within the laser processed squares and in unprocessed protein film using Bruker Senterra microscope-spectrometer (laser excitation at 785 nm). Registered Raman spectra were processed and the autofluorescence background was subtracted using Origin Pro 2015 software. Infrared spectra were also registered locally using the Bruker Lumos II Fourier-transform infrared (FTIR) microscope-spectrometer in an attenuated total reflectance mode.

Results and discussion

Upon laser processing the surface layer of protein film was partially ablated and at the same time the laser-processed squares became fluorescent and were readily observed on the fluorescent microscopy images (Fig. 1, *b*). The fluorescence intensity within the squares increased with the processing laser power and for 10 mW power it was several times stronger than intensity of autofluorescence of the BSA protein (Fig. 2, *a*). The effect of varying the laser power was threshold- like: at the minimal power (2 mW) the film was ablated, but the protein autofluorescence was bleached, and no new fluorescence was generated. As a result, the fluorescence intensity in the processed square became weaker than the autofluorescence background. Above the threshold power the emission spectral shape did not depend on the processing laser power. At the same time emission spectra in the processed squares were somewhat redshifted compared with the autofluorescence spectrum of the BSA. This indicates that autofluorescence and laser-generated fluorescence in the BSA originates from different fluorophores. Emission spectra of processed BSA were excitation- dependent and shifted to the red area with increase of the excitation wavelength from 405 to 561 nm (Fig. 2, *b*). The emission maxima shifted from 540 to more than 600 nm when changing the excitation wavelength from 488 to 561 nm (Fig. 2, *c*). The emission was the brightest under the blue (405 nm) excitation.

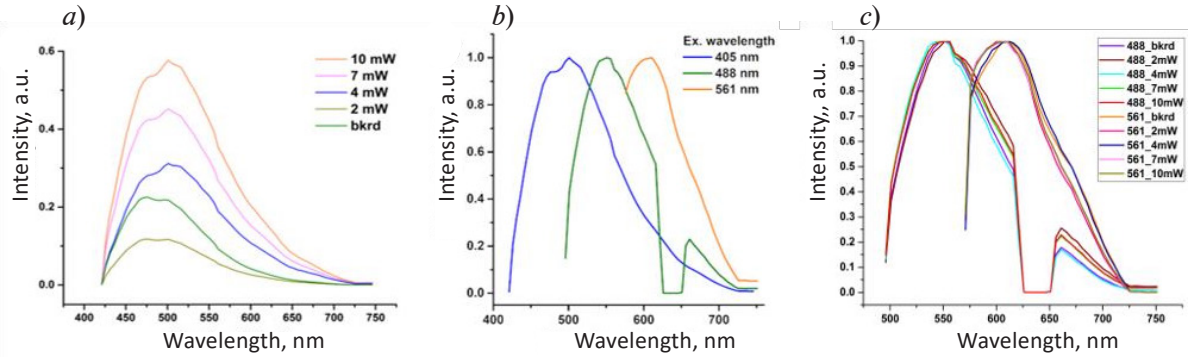

Fig. 2. Averaged fluorescence emission spectra of laser-processed BSA as a function of laser power compared with the background from the unprocessed BSA. Excitation at 405 nm (*a*); comparison of the emission spectra in the laser-processed square under 405, 488 and 561 nm excitation at laser power of 10 mW (*b*); comparison of normalized emission spectra of laser-processed squares at 488 and 561 fluorescence excitation for all laser regimes (*c*)

Multiple scanning cycles of the same area at a fixed laser power led to brighter fluorescence (Fig. 1, *b*), indicating accumulation of fluorescent products of laser irradiation. Fluorescence intensity monotonically increased with a number of cycles. The dependence on the number of cycles was not linear and could be approximated with a function $I = I_{auto} + I_0 \ln(N + 1)$, where I_{auto} is autofluorescence intensity of the normal BSA, N is the number of scanning cycles, I_0 is a constant (Fig. 3, *b*).

FTIR spectra of the protein revealed characteristic amide bands at 3280, 1650, 1535, and 1300 cm^{-1} as well as vibrational band associated with alkyl and carboxylate groups near 2900, 1450 and 1395 cm^{-1} (Fig. 4, *a*). Laser-processed BSA exhibited the same bands of similar shape and intensity. The largest difference was a weaker intensity in the OH stretching range (3000–3600 cm^{-1}), probably due to evaporation of residual water within the protein film. Similarity of the FTIR spectra demonstrates that the chemical structure of the protein underwent little changes after the laser

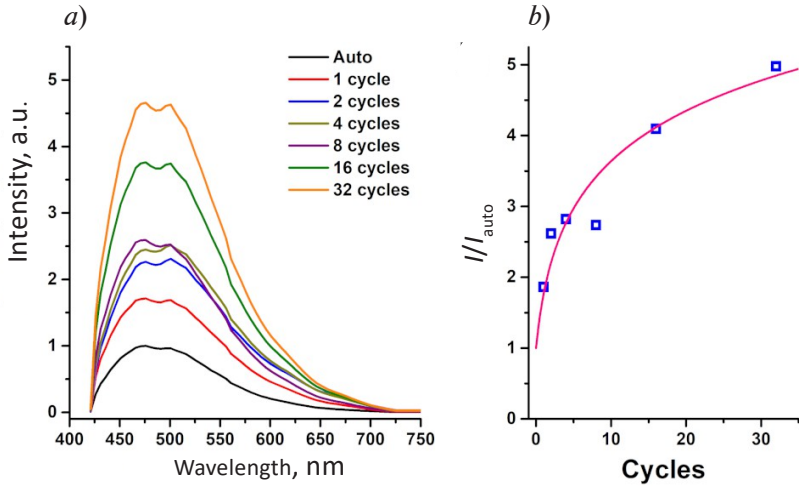

Fig. 3. Fluorescence emission spectra of the laser-processed BSA with a different number of scanning cycles, 5 mW laser power. Autofluorescence spectrum of the unprocessed BSA is presented for comparison (black line). Excitation at 405 nm (*a*). Ratio of the integral fluorescence intensity in the processed square to the autofluorescence intensity of unprocessed BSA protein as a function of the number of scanning cycles (squares) and its fit with a logarithmic function (*b*)

processing. More specifically, unaltered maxima and shapes of the Amide I and III bands suggest that the secondary structure of the protein was not significantly altered. Similarly, Raman spectra ofnormal and laser-processed proteins demonstrated that no conspicuous new vibration bands appeared after the laser irradiation (Fig. 4, *b*). In particular, absence of characteristic G and D bands of carbon near 1580 and 1310 cm^{-1} indicates that no appreciable carbonization of the protein occurred.

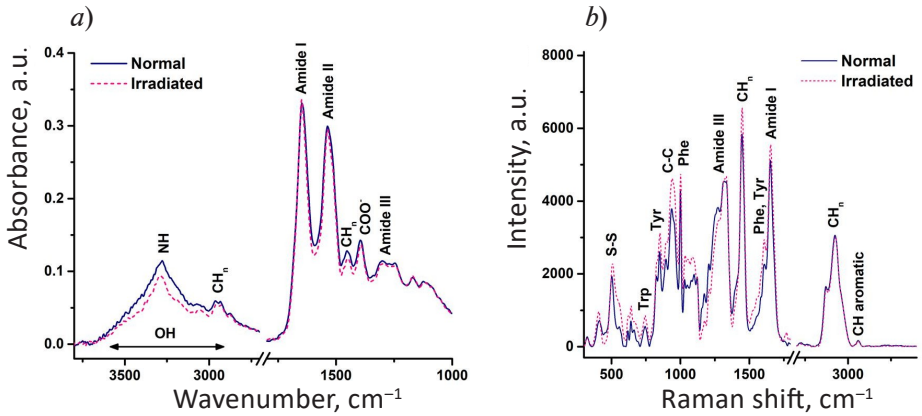

Fig. 4. Comparison of the FTIR (***a***) and Raman (*b*) spectra of the normal BSA protein and laser-processed BSA (at 10 mW)

A possible mechanism of fluorescent patterning of an organic material, as demonstrated for cellulose, is a generation of fluorescent carbon dots through laser-induced thermal carbonization [6]. In our work FTIR and Raman analysis excludes large-scale carbonization of the protein and does not support carbonization mechanism of fluorescence generation. Thus, fluorescence enhancement resulted from relatively minor changes in chemical composition or physical characteristics of the BSA. We hypothesize that these changes might include densification of the protein as a result of the laser ablation, leading to enhancement of its nonconjugated fluorescence [10]. Laser-induced oxidation of the aromatic residues of the protein mediated by nonlinear absorption of laser pulses, in particular production of fluorescent kynurenine and N-formylkynurenine from tryptophan, offers another possible mechanism [8].

Conclusions

We demonstrated that femtosecond laser ablation of the solid protein film produces microstructures with multicolor and excitation-dependent visible fluorescence. Processing laser power and exposure enable control of the fluorescence intensity. Intensities several times stronger than the autofluorescence background can be achieved, so that micropatterns can be readily discerned on fluorescent images. Infrared and Raman analysis indicate only minor changes in chemical structure of the protein as a result of laser irradiation.

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Received 29.09.2025. Approved after reviewing 22.10.2025. Accepted 22.10.2025.