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Fluorescent cell imaging with femtosecond laser pulses-produced protein nanoaggregates

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Abstract. We demonstrated formation of fluorescent products from model protein - bovine serum albumin, induced by femtosecond laser irradiation and characterized optical properties of these products. Laser-modified BSA showed relative biocompatibility and can be employed for cell imaging.

Keywords: proteins, femtosecond laser pulses, nanomaterials, fluorescence, bioimaging

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Флуоресцентная визуализация клеток с применением белковых наноагрегатов, полученных методом лазерного синтеза

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Аннотация. В работе исследована возможность агрегации бычьего сывороточного альбумина с помощью фемтосекундного лазерного излучения с последующей оценкой флуоресцентных характеристик продуктов синтеза. Также была продемонстрирована биосовместимость продуктов синтеза и возможность их применения для имиджинга на клеточной линии A549.

Ключевые слова: белки, фемтосекундные лазерные импульсы, наноматериалы, флуоресценция, биоимаджинг

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Introduction

Femtosecond laser synthesis of fluorescent nanoproducts from biomolecules is an intriguing phenomenon, which can be exploited in novel approaches to bioimaging [1–2]. Previously we demonstrated laser synthesis of fluorescent carbon dots from essential amino acids [3]. As a next step we study products obtained from a model protein bovine serum albumin (BSA). It is known that albumin can form aggregates, which unlike the monomer protein emit bright visible fluorescence [4–5]. We examined if the femtosecond laser irradiation can trigger similar aggregation of BSA and formation of fluorescent products and studied their optical properties. We also probed biocompatibility of BSA products and their employment as fluorescent cell imaging agents.

Materials and Methods

Synthesis and characterization of products. Aqueous solution of BSA (1 mL, 0.1 g/mL) in a glass vial was irradiated with trains of amplified femtosecond laser pulses focused by a spherical lens (f = 8 mm, 0.5NA). Central wavelength of laser pulses was 1033 nm, repetition rate was 50 kHz, duration was 250 fs, pulse energy was 60 μJ. (Fig. 1)

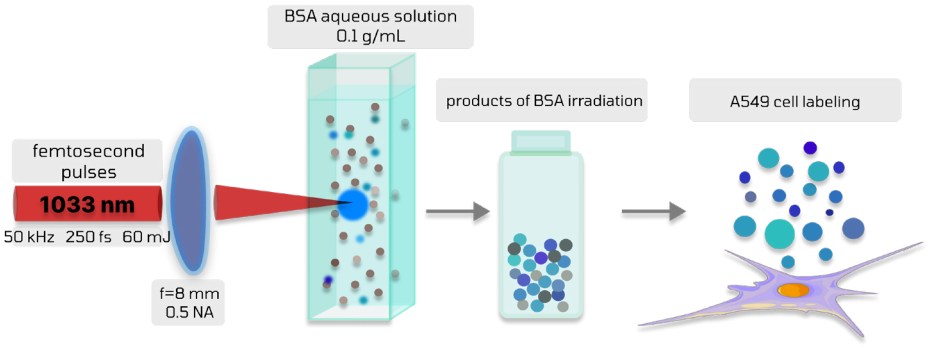

Fig. 1. Experimental setup

Absorption and photoluminescence spectra of irradiated BSA in water were recorded with Shimadzu spectrophotometer (UV-3600) and spectrofluorometer (RF-5031 PC). Fluorescence quantum yield (356 nm excitation) was estimated with the slope method using ethanol solution of anthracene as a standard (quantum yield = 0.27). Fluorescence decay and fluorescence anisotropy decay kinetics in aqueous solution were registered with pulsed laser excitation at 360 nm at 450 nm emission wavelength using time-correlated single-photon counting module (SPC-150N, Becker&Hickl GmbH). Protein aggregates sizes were analyzed with dynamic light scattering (DLS) technique using a Photocor Compact-Z setup.

Labeling of living A549 cells. A549 cells (lung adenocarcinoma) were incubated at 37 °C in 5% CO_2 humidified atmosphere with CDs dissolved in medium in the ratios of 1:200, 1:40 and 1:8 for 12 hours. Fluorescent images and lambda scans of the cells after labeling were obtained using a laser scanning confocal microscope Zeiss LSM 980 (Carl Zeiss Microscopy, Jena, Germany), 20x Plan-Apochromat objective (NA = 0.8). One-photon excitation was performed with a 405 nm laser. Fluorescence was recorded at 420–750 nm range.

A549 cell viability test. A549 cells (lung adenocarcinoma) were incubated at 37 °C in 5% CO_2 humidified atmosphere with CDs dissolved in medium in the ratios of 1:200, 1:40 and 1:8 for 2 hours. After that, cells were stained with acridine orange and propidium iodide to identify dead cells at a concentration sufficient for imaging, but optimal for minimizing risks of cell death after staining. Confocal imaging was obtained immediately after staining without washing of samples. Images of 3 randomly chosen regions from each sample were obtained and used for cell counting in Fiji software. Ratios of dead/alive cells (nuclei stained with PI and AO respectively) were counted to estimate cell viability in each sample.

Results and Discussion

Aqueous solution of the normal BSA was almost non-absorbing above 300 nm and emitted only weak visible fluorescence. Laser irradiation resulted in yellowing of the BSA solution (Fig. 2, *a*) and emergence of broadband optical absorption in the near-ultraviolet and visible range with extinction coefficient of the order of 10^4 $M^{-1}cm^{-1}$ (Fig. 2, *b*). Laser-irradiated BSA solution emitted bright fluorescence upon excitation in the near-ultraviolet and visible range. Its intensity was many times stronger than fluorescence intensity of normal BSA.

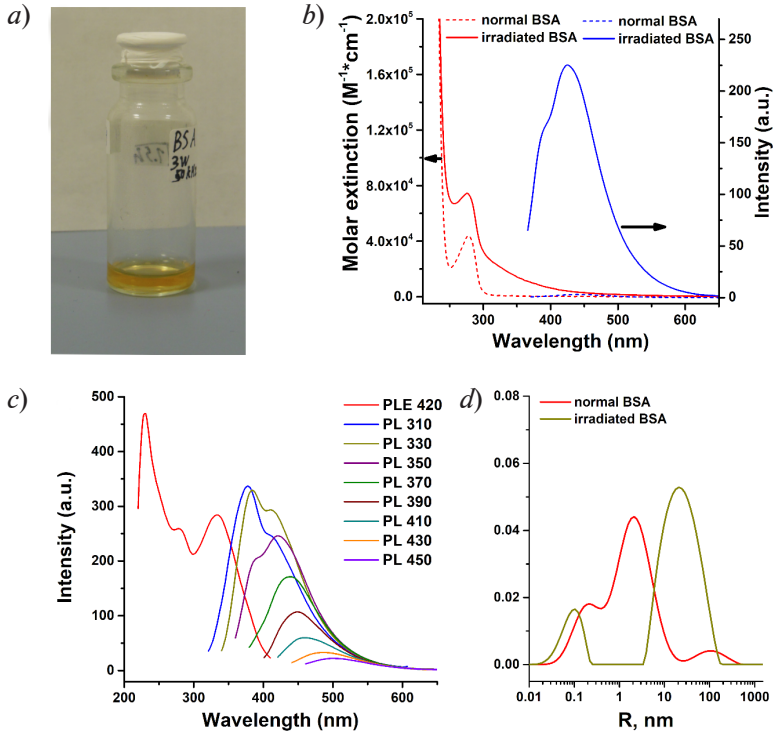

Fig. 2. Image of laser-irradiated BSA sample (*a*). Absorption and fluorescence spectra of normal (dashed lines) and laser-irradiated (solid lines) BSA. Fluorescence excitation at 350 nm (*b*). Photoluminescence excitation spectrum (PLE) and emission spectra (PL) with excitation wavelength ranging from 310 to 450 nm (*c*). Distribution of hydrodynamic radii of normal and laser-irradiated BSA obtained from the DLS (*d*)

Photoluminescence excitation spectrum, registered at 420 nm emission wavelength, revealed three distinct absorption bands at 230, 280 and 330 nm. The band at 280 nm corresponds to excitation of intrinsic luminescence of aromatic residues of the BSA (tyrosine and tryptophan), whose emission peaks at 340 nm, but also produces a relatively weak tail in the visible range. This band is also seen at the absorption spectrum (Fig. 2, *c*). The bands at 230 and 330 nm are attributed to new chromophores formed in the BSA by laser irradiation. Fluorescence spectra were dependent on the excitation wavelength: their shape and peak position changed with a shift of the excitation wavelength (Fig. 2, *c*). When excited near 350 nm the emission peak was at 420 nm with the corresponding fluorescence quantum yield equal to 7.4%, and the fluorescence lifetime to 1.35 ns.

Dynamic light scattering demonstrated formation of aggregates in the irradiated BSA solution with the mean radius of about 30 nm of tens nm – almost an order of magnitude larger than hydrodynamic radius of normal BSA in water (3.9 nm) [6] (Fig. 2, *d*). Thus, these aggregates consist of a large number (at least hundreds) of protein monomers. Human and bovine serum albumin are known to form fluorescent oligomers in concentrated solutions [4–5]. We suggest that femtosecond laser irradiation produces protein aggregates, which remain stable even in diluted solution and these aggregates are a likely source of enhanced absorption and fluorescence of irradiated BSA.

Brightest fluorescence of CDs was observed in group with highest concentration of CDs. Lambda scanning of cells after incubation with CDs during 12 hours demonstrated broadening of dominant emission peak. Emergence of shoulder at 430–450 nm range can be a mark of CDs uptake by cells (Fig. 3).

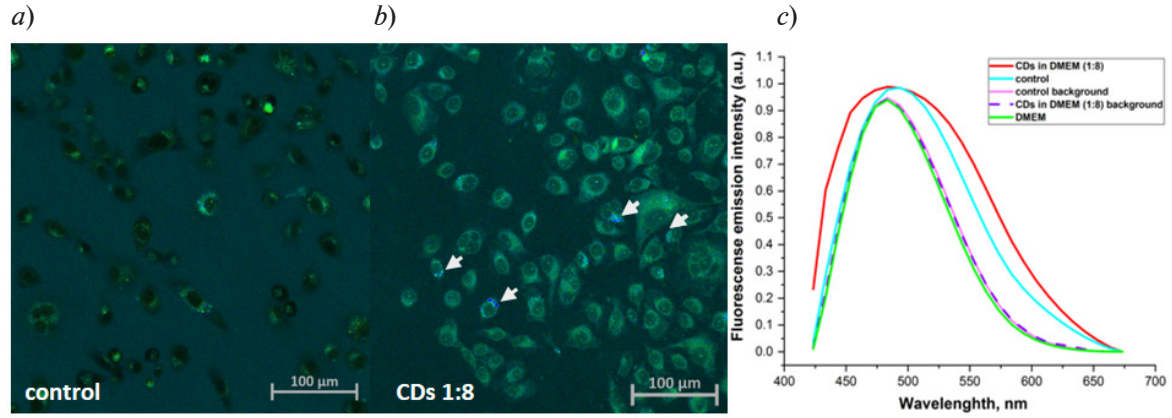

Fig. 3. Lambda scanning of A549 cells, incubated with CDs at ratio of 1:8 for 12 hours. Scanned areas are pointed by arrows. The most representable one was taken for the graph. Lambda stack of cells from control group (*a*), lambda stack of cells, incubated with CDs at ratio of 1:8 for 12 hours (*b*), fluorescence emission distribution (*c*)

Confocal imaging also revealed formation of granules (probably vesicles) in cytoplasm with fluorescence in blue spectral diapason. (Fig. 4).

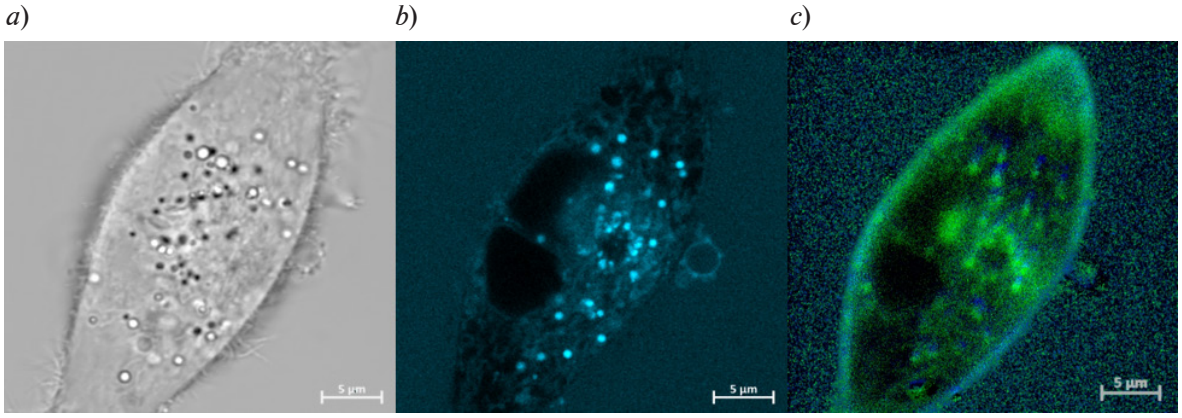

Fig. 4. Confocal images of individual A549 cell, incubated with CDs at ratio of 1:40. Bright field image of the cell (*a*), confocal fluorescent image at 405 nm wavelength (*b*), lambda stack and fluorescence distribution within the cell (*c*)

Short-time labelling of A549 cells with CDs showed an increased percentage of dead cells in all experimental groups. Cell viability was not dose-dependent, quantity of dead cells is almost the same for all concentrations of CDs. (Fig. 5). Gradually reducing ability of cells to attach substrate

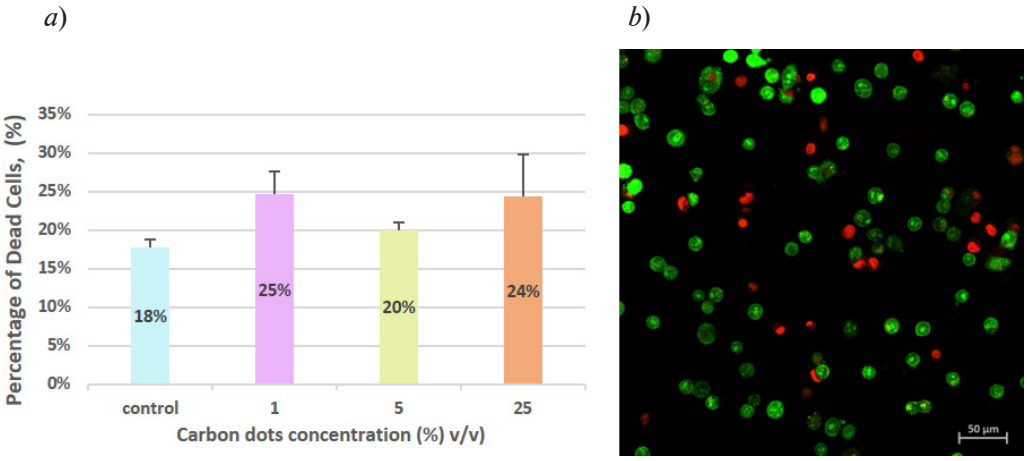

Fig. 5. A549 cell viability test (*a*), confocal image of A549 cells, stained with acridine orange (live, green) and propidium iodide (dead, red) (*b*)

was observed and was proportional to concentration of CDs. Cells from the sample with highest concentration remained in suspension after 12 hours of incubation. Impossibility of cell to attach substrate can be a trigger of cell death. It is also known, that sensibility of cells to nanoparticles depends on a cell cycle phase. Exact mechanism of cell death experienced by cells under BSA-based CDs uptake remains to be studied.

In conclusion, we found that femtosecond laser irradiation enhances absorption and visible fluorescence of the BSA solution and produces nanoscale protein aggregates. We demonstrated applicability of laser produced protein aggregates for cell fluorescent imaging.

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