Mass spectrometry analysis of C-dots produced by femtosecond laser irradiation of L-lysine film.

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Abstract. The production of carbon dots (C-dots) by femtosecond lasers within living cells and tissues is a novel approach, which has a great potency for intracellular bioimaging. An exact mechanism of fluorescent particles production as well as their composition still remains unknown. In this work we use L-lysine film as a model system to study the mechanism and the composition of C-dots produced by femtosecond laser irradiation investigated by time-of-flight secondary ion mass spectrometry (ToF-SIMS).

1. Introduction
Fluorescent bioimaging is the key method of living cell structure and functioning investigation. Usually bioimaging requires exogenous fluorophore delivery or insertion of genes, coding special fluorescent proteins. A novel approach for bioimaging has been proposed recently [1-3], which is based on the generation of fluorescent carbon dots (C-dots) within a cell or a tissue from its material by femtosecond laser. But chemical-physical processes, leading to C-dots formation, are poorly described and debatable. One research concluded that fluorescent particles are produced as a result of thermally-induced Maillard reaction from proteins and amino acids [4]. Our results indicate that this fluorescent synthesis implies local carbonization and graphitization of the biomaterial [2, 5], induced by laser-generated plasma [6]. However, both processes are possible or a consequence of biomolecule conversion can take place, and their contribution depends on the irradiation parameters.

Complexity and heterogeneity of the chemical composition of cells and tissues make it difficult to analyse the laser synthesis processes of fluorescent products. In this work we used a film made of L-lysine amino acid as a model system to investigate laser synthesis of C-dots. Being subjected to intensive femtosecond laser radiation, L-lysine is known to be an effective precursor for fluorescent species C-dots synthesis. L-lysine film, exposed to 80 MHz and 50 kHz 800 nm femtosecond laser, was then analysed by ToF-SIMS.

2. Methods

2.1. L-lysine film preparation
10 μl of L-lysine solution (0.5 g/l) was placed at the cover glass (24*24 mm, Heinz Herenz), previously subjected to UV lamp (185 and 254 nm), and then covered with another cover glass, which
was not subjected to UV. This “sandwich” was dried for 24 hours at 40°. Then the upper cover glass was removed, and the smooth L-lysine film remained at the lower glass.

2.2. Laser parameters
Femtosecond laser pulses at 790 nm central wavelength, 40 fs duration and 80 MHz repetition rate were generated by a Ti:sapphire oscillator (Tsunami, Spectra-Physics). Pulses could be optionally frequency-doubled in a nonlinear crystal or their repetition rate could be decreased to 50 KHz by a pulse-picker (Avesta Project). Laser pulses trains were coupled to an optical microscope (Olympus IX71) and focused by an objective lens (40X, 0.7NA). L-lysine film was placed on the microscope sample stage and scanned across the focused laser beam using a piezoelectric scanner (NT-MDT).

2.3. Parameters of irradiation
In these experiments we used following parameters for the irradiation of different areas of L-lysine film: (1) \( \lambda = 790 \text{ nm}, \nu = 50 \text{ kHz}, \) pulse energy 6 nJ, the area has been irradiated 10 times, area size is 100\(\times\)100 \(\mu\text{m}\), 80\(\times\)80 points; (2) \( \lambda = 790 \text{ nm}, \nu = 50 \text{ kHz}, \) pulse energy 6 nJ, the area has been irradiated only once (single irradiation), area size is 100\(\times\)100 \(\mu\text{m}\), 80\(\times\)80 points; (3) \( \lambda = 790 \text{ nm}, \nu = 80 \text{ MHz}, \) pulse energy 1.9 nJ, single irradiation, area size is 80\(\times\)80 \(\mu\text{m}\), 30\(\times\)30 points; (4) \( \lambda = 390 \text{ nm}, \nu = 80 \text{ MHz}, \) pulse energy 1.3 nJ, single irradiation, area size is 100\(\times\)60 \(\mu\text{m}\), 40\(\times\)40 points; (5) \( \lambda = 390 \text{ nm}, \nu = 80 \text{ MHz}, \) pulse energy 0.17 nJ, single irradiation, area size is 100\(\times\)60 \(\mu\text{m}\), 40\(\times\)40 points. We used 40x objective with NA = 0.55.

2.4. Mass spectrometry imaging by ToF-SIMS
L-lysine film was analyzed by mass spectrometer ToF-SIMS 5 (ION-TOF, Germany). Bi\(^{+}\) cluster ions were used as primary ions. Primary ions dose density was \( 4 \times 10^{11} \text{ ions/cm}^2 \). Scanning area was 400 \( \times \) 400 \( \mu\text{m} \) with pixels size \( \sim 0.8 \mu\text{m} \). Chemical maps were obtained for both positive and negative secondary ions.

2.5. Fluorescent spectra measurement
Fluorescence of L-lysine film was locally excited by two-photon absorption of femtosecond laser pulses (790 nm, 40 fs, 80 MHz, 0.5 nJ) focused by the microscope objective lens, then collected by the same lens, coupled to the monochromator (Acton SP300i), and its spectra were registered by an intensified CCD camera (PI MAX 2, Roper Scientific).

2.6. Confocal imaging
Confocal images were obtained by LSM 980 confocal system (Carl Zeiss Microscopy, Jena, Germany), 20\(\times\) Plan-Apochromat objective (NA = 0.8). Excitation of the fluorescence was performed by one-photon mode by 405 nm laser. Emission recorded at 450/600 nm range.

3. Results
Fluorescent products were formed within each area after femtosecond laser irradiation (figure 1). Fluorescent spectra of irradiated and non-irradiated L-lysine film are shown at figure 2 and it has maximum at nearly 510 nm.

The chemical composition of irradiated L-lysine film was studied by ToF-SIMS mass spectrometer. Figure 3 represents chemical maps of irradiated areas for different secondary ions. Lysine (C\(_6\)H\(_{15}\)N\(_2\)O\(_2\)) signal (d) decreases for each irradiated area. The lowest signal was observed in the area 1, which corresponds to the highest irradiation dose. NH\(_3\) yield (b) dropped in the area 1 as well, which is likely caused by nitrogen splitting off.

Lysine and NH\(_3\) redistribution was clearly observed in the areas 3 and 4 and could evidence lysine diffusion under laser irradiation. Interestingly, sodium signal increased in the area 1. One may suggest that sodium diffused into the film from the surface of substrate (cover glass). However silicon signal was not detected hence the film in the area 1 was not evaporated completely. Ion with m/z = 177 also showed increase yield in the area 1. This ion may be attributed to C\(_{12}\)H\(_7\)O\(^\text{+}\) - the product of lysine
dimerization. On the contrary ion with m/z = 171 (possibly C₈H₁₇N₃O⁺, C₈H₁₇NO₂⁺ or C₉H₁₇N₃O₂⁺) showed increase intensity in areas 2, 4 and 5.

![Image](image.png)

**Figure 1.** Confocal imaging of irradiated L-lysine film. A – transmitted light image, B – fluorescent image. Numbers indicate areas with different parameters of laser irradiation. Non-irradiated L-lysine is around areas 1-5.

![Image](image2.png)

**Figure 2.** Fluorescence spectra of irradiated and non-irradiated L-lysine.

Alterations in other ion distributions were also observed. The signal of the ion m/z 44 (CO₂⁻) dropped in irradiated fields, and the most intensive decrease was observed in the square 1. The intensity of the ion with m/z = 168 is in a good correlation with the irradiation dose, so it is probably the final product of the laser irradiation. Possible formula of this ion are: C₇H₈N₂O₃⁺, C₁₀H₆N₃⁺, C₁₂H₄O⁺, C₈H₅O⁺. As well, ions C₃ (m/z = 48), C₆H₅⁻ (m/z = 77) and C₇H₇⁺ (m/z = 91) had an increase in irradiated L-lysine.
Figure 3. Mass spectrometry imaging of L-lysine film irradiated by femtosecond laser. Numbers indicate areas with different parameters of laser irradiation. A - optical image; B - NH$_3^+$ distribution, C - Na$^+$ distribution, D - lysine molecular ion (C$_6$H$_{15}$N$_2$O$_2^{2+}$) distribution, E - distribution of ion with m/z = 171, F - distribution of ion with m/z = 177.

4. Discussion
An average fluorescence intensity in square 1 seems to be lower than in square 2 is spite of 10 times higher irradiation dose (Figure 1, b). It can be explained by photobleaching of fluorophores, produced at the first irradiation, during subsequent irradiations. Thereby, the significant lysine signal decay (figure 3, d) is not a consequence of C-dots formation. The fact that with an increase in the duration of the C-dots synthesis reaction or an increase in the synthesis temperature, the products became less fluorescent, is in a compliance with our results.

C-dots formation under the action of second harmonic laser radiation ($\lambda = 390$ nm) is also possible, but 390 nm radiation has lower order of nonlinear absorption. It is clearly visible by melting of 4 and 5 squares (figure 1).

L-lysine dimers and oligomers are not fluorescent. Fluorescence of irradiated L-lysine could occur as a result of carbonization and aromatization leading to carbon dots-like products. It is confirmed by C$_4$ (m/z = 48), C$_6$H$_3^+$ (m/z = 77) and C$_7$H$_7^+$ (m/z = 91) ions signal increase. Although it is difficult to accurately identify the final product at the moment, it should be noted that the position of mass-spectrometry peaks indicates either a very small amount of hydrogen relative to carbon, or a noticeable content of oxygen and / or nitrogen. This most likely indicates the occurrence of carbonization and oxidation processes during irradiation, and oligo- and poly- L-lysine could be the intermediate products, from which C-dots are produced.

5. Conclusion
C-dots formation from L-lysine film is possible for both 790 and 390 nm femtosecond laser radiation, but 790 nm allows to produce C-dots more precisely. The mass-spectrometry analysis has performed that greater yield of L-lysine polymerization products was achieved by the laser radiation with higher pulse energy. Carbonization and aromatization can be involved in the process of C-dots formation.
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