Femtosecond laser synthesis and comparative analysis of fluorescent carbon dots from L-lysine aqueous solution

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Abstract. Laser synthesis of fluorescent species from biomolecules in living cells and tissues offers unique capabilities for fluorescent bioimaging, yet little is known about its mechanisms and characteristics of products. We examine synthesis of fluorescent products from water solution of L-lysine upon irradiation by trains of femtosecond laser pulses with varying parameters. We demonstrate that irradiation products contain nanoscale carbon-based fluorescent particles (carbon dots) with multi-colour and excitation-dependent emission. Morphology, chemical composition and fluorescent characteristics of irradiation products strongly depend on laser pulses parameters.

1. Introduction
Production of fluorescent species in living cells and tissues by femtosecond laser irradiation is a novel approach to fluorescent bioimaging which offers a unique advantage of non-invasive, rapid, highly-localized and targeted fluorescent labelling [1-2]. Exact mechanisms of laser synthesis and chemical nature of fluorescent products remain debatable [2-3] and their analysis is complicated by heterogeneity of cells and tissues and difficulty of isolating irradiation products. A possible role of synthesis of fluorescent carbon dots (CDs) in laser production of fluorescent species was highlighted recently [2,4].

In order to clarify synthesis mechanisms and characteristics of products we examined femtosecond laser production of fluorescent species from a model biological molecule. A common proteinogenic amino acid L-lysine, which is also highly effective as precursor for hydrothermal or microwave synthesis of CDs, was chosen as a model compound. In order to understand the role of various physicochemical effects of nonlinear absorption and the influence of laser parameters the water solution of L-lysine was irradiated with femtosecond laser pulses in three different regimes: one corresponding to laser breakdown in aqueous media and two sub-breakdown regimes with different laser wavelength, and comparative analysis of irradiation products was performed using a combination of optical spectroscopy, energy-dispersive X-ray spectroscopy, TOF-SIMS mass spectroscopy, and FTIR spectroscopy.

2. Materials and methods

2.1. Laser synthesis and isolation of products.
Lysine solution of 500 mg/mL in water (Sigma, HPLC) was prepared from L-lysine powder (Sigma). 100 µL of the solution was placed in 100 µL quartz cuvette for irradiation by femtosecond pulses at 80 MHz repetition rate and 2 mL was placed into 10 mL serum vial for irradiation at 1 kHz repetition rate.
rate. Lysine solutions were irradiated in 3 regimes: 1) femtosecond laser pulses at 1 kHz repetition rate, wavelength of 800 nm, 1.4 mJ pulse energy, 50 fs pulse duration and 20 h irradiation time; 2) femtosecond laser pulses at 80 MHz repetition rate, wavelength of 740 nm, 1.8 W average optical power, 50 fs pulse duration and 70 h irradiation time; 3) femtosecond laser pulses at 80 MHz repetition rate, wavelength of 370 nm (SHG), 0.3 W average optical power, 100 fs pulse duration and 70 h irradiation time, resulting in samples referred as 1-3. After irradiation samples were dialyzed for 72 hours in 2,000 MWCO dialysis units in order to remove unreacted L-lysine.

2.2. Samples characterization

UV-VIS absorption and photoluminescence spectra of aqueous solutions of 1-3 in a 3.8-ml quartz cuvette were recorded with Shimadzu UV-3600 spectrophotometer and Shimadzu RF-5031PC spectrophotometer. Fluorescence quantum yields of 1-3 solutions in ethanol were estimated by the slope method using excitation at 356 nm wavelength and anthracene solution in ethanol (Φ = 27%) as a fluorescence standard. For measurement of fluorescence anisotropy decay of aqueous solutions of 1-3 we recorded two fluorescence decay curves I∥(t) and I⊥(t) for emission with polarization parallel and perpendicular to the excitation laser. Fluorescence was excited with frequency-doubled femtosecond laser pulses (355 nm central wavelength, 100 fs pulse duration). Decay curves were recorded at 450 nm emission wavelength with a Beckr-Hickl SPC-150N time-correlated single photon counting system. The fluorescence anisotropy was calculated by the formula r(t)=(I∥−α(λ)I⊥)/[I∥+2α(λ)I⊥], where α is the empirical correction factor. The average hydrodynamic volume of luminescent particles was estimated by the formula: \( V = k_B T \tau_{rot} / \eta \), where \( \tau_{rot} \) is the characteristic anisotropy decay time, \( k_B \) is the Boltzmann constant, \( T \) is the temperature, and \( \eta \) is the dynamic viscosity of the water.

Samples diluted in ethanol and dried on a cover slip were analyzed with an atomic-force microscopy unit (SMENA-B, NT MDT) in an intermittent contact mode. FTIR infrared spectra were recorded using the Lumos I FTIR microscope-spectrometer in attenuated total reflection (ATR) mode from a drop of water solution dried on a surface of a CaF₂ window. Elemental analysis with energy-dispersive X-ray spectroscopy was performed using a Prisma-E electron microscope (Thermo-Fisher), using L-lysine with a known C:N:O ratio (3:1:1) as a calibration sample. Mass spectroscopy analysis of dried samples on borosilicate cover slip in positive and negative ions was performed with TOF.SIMS 5 mass spectrometer (ION-TOF). Mass spectra of l-lysine film on a cover slip were used as a reference.

3. Results and discussion

Exposure to femtosecond laser pulses resulted in yellowing of originally colorless lysine solution and appearance of appreciable visible luminescence in all irradiation regimes. Analysis of UV-Vis absorption spectra demonstrated a large difference between samples. 1 absorbed mostly in the UV region with a conspicuous shoulder at 330 nm and a weak absorption tail above 400 nm, 2 had appreciable absorbance only in the deep UV region, 3 had a strong absorption peak at 315 nm, but very small visible absorption (Figure 1). Absorption features in the near-UV and visible range demonstrates effective formation of chromophores with conjugated systems in 1 and 3, and the kilohertz irradiation regime was especially effective for producing chromophores absorbing in the visible range.

All three samples exhibited multicomponent near-UV and visible photoluminescence with at least two separate emission peaks: a near UV peak with an emission maximum at ca. 380 nm and excitation maximum at 310 nm, and a blue peak with emission maximum at 420-430 nm and excitation maximum at ca. 350 nm. The second peak was excitation-dependent: its maximum shifted to a longer wavelength with increase of the excitation wavelength parallel to the attenuation of emission intensity.

Such excitation dependence is typical for photoluminescence of carbon nanodots [5] and is interpreted as a result of multicomponent character of their emission [6, 7]. Importantly, the proportion between emission components was different, and 1 had the strongest visible luminescence component, whereas 3 – the strongest near-UV component.
Figure 1. Absorbance, photoluminescence emission (PL) and photoluminescence excitation (PLE) spectra of aqueous solutions of 1 (a), 2 (b) and 3 (c).

Additionally, under 470 nm excitation exhibited a distinct green emission peaks with emission maximum at ca. 510 nm. Estimated quantum yield of the blue luminescence component was small for all the three samples, the highest yield of 6% belonged to 1. In summary, femtosecond laser irradiation produced several fluorescent moieties from L-lysine, their composition and relative content was strongly dependent on the irradiation regime, and kilohertz irradiation was the most effective for producing moieties with visible luminescence.

In order to remove unreacted L-lysine for further chemical analysis the samples 1-3 were subjected to dialysis, which also removed irradiation products with a molecular mass smaller than appr. 2 kDa. This removal resulted in attenuation of absorption and luminescence intensity which was the strongest for 3 and the weakest for 1 (Figure 2a). This indicates that absorption and photoluminescence was only partly attributable to products with large molecular mass (nanoparticles, oligomers, aggregates etc), while a large part of luminescence belonged to smaller molecular weight fragments which were removed by dialysis. From the attenuation ratio it can be concluded that the kilohertz regime (1) produced products with the largest mass, whereas the second harmonic irradiation (3) – with the smallest. Absorption and photoluminescence spectra of 1 were virtually unchanged by dialysis, whereas for the 2 and 3 these changes were strong and their spectra became nearly identical (Figure 2b). UV-Vis spectra of the dialyzed 2 and 3 were similar to 1 with a characteristic absorption shoulder at ca. 320 nm and a tail above 400 nm. The relative strength of the near-UV luminescence peak at 380 nm also strongly decreased. Thus, UV-emitting fluorophores in 2-3 tended to be associated with relatively small molecules or aggregates, whereas fluorophores with blue and green emission - with heavy weight products. We further elucidated characteristics of dialyzed samples 1-3 using measurements of emission anisotropy kinetics in ethanol solution under pulsed laser excitation at 356 nm (Figure 2c). Anisotropy decay from an initial value of approximately 0.3 on a nanosecond and sub-nanosecond time scale is attributed to rotational diffusion of fluorescent moieties, which changed orientation of the emission dipole with time and depolarized emission. Characteristic rotation time calculated from the multiexponential peak yields effective hydrodynamic volumes of 1-3 as 10.6, 0.7 and 1.4 nm³ respectively. The last two values are unexpectedly small, since particles with volumes smaller than appr. 2.5 nm³ can pass through pores of the dialysis membrane and are mostly filtered out during the dialysis.

We explain anomalously fast rotational diffusion with a flexible structure of heavy-weight products in 2-3, e.g. flexible polymer chains, where segments bearing fluorophore can change orientation independently of other segments, leading to fast depolarization (Figure 2d). By a contrast, 3 has
fluorophores are bound to rigid nanoparticles and can change orientation only with rotation of the nanoparticle as a whole which occurs on the scale of nanoseconds.

Figure 2. (a) Percentage of the original PL intensity remaining after dialysis for 1-3, excitation at 350 nm. (b) Absorbance, PLE and PL spectra of the dialyzed samples 2-3. (c) Emission anisotropy decay kinetics of 1-3 in aqueous solution. Excitation with femtosecond laser pulses at 356 nm. (d) Proposed structure of heavy-weight luminescent products in 1-3.

According to the EDS measurements (Table 1) the content of carbon in irradiation products was larger than in l-lysine, especially in 1, which indicates an onset of laser-driven carbonization. The O:N ratio was larger than 1:1 in l-lysine. Additional oxygen apparently resulted from oxidation of products during the irradiation in presence of air dissolved in aqueous solution.

Table 1. Elemental composition of 1-3 compared with L-lysine.

| Sample | C (at. %) | N (at. %) | O (at. %) |
|--------|----------|----------|----------|
| L-lysine | 60       | 20       | 20       |
| 1      | 67.6     | 14.5     | 17.9     |
| 2      | 62.1     | 16.4     | 21.5     |
| 3      | 61.1     | 17.2     | 22.4     |

FTIR spectra demonstrate that chemical composition of 2-3 was similar and at the same time much different from 1 (Figure 3a). Spectrum of 1 included typical amide peaks (amide I peak at 1630 cm$^{-1}$, amide II peak of secondary amides at 1560 cm$^{-1}$, amide III peak of primary amides at 1390 cm$^{-1}$), which indicates that formation of heavy weight products under kilohertz irradiation proceeded via polymerization of l-lysine. Curiously, presence of primary amides indicates that the resulting polylsine nanoparticles underwent reorganization, typical for carbon dots formation in polar solvent, which transferred amide groups from the interior to the nanoparticle surface [8]. CH$_2$ stretching and bending peaks at 2930, 2860 and 1450 cm$^{-1}$ show presence of alkyl chains derived from l-lysine, meaning that carbonization of nanoparticles was incomplete. Broad OH and CO stretching peaks at 3400 and 1070 cm$^{-1}$ indicate that oxidation of nanoparticles resulted in formation of surface hydroxyl groups. 2 and 3 exhibited strong asymmetric and symmetric bending vibrations of NH$_3^+$ at 1605 and 1510 cm$^{-1}$, asymmetric and symmetric stretching vibrations of COO$^-$ at 1580 and 1405 cm$^{-1}$, a broad NH$_3^+$ stretching peak at 3000 cm$^{-1}$ and COO$^-$ bending at 620 cm$^{-1}$, which are characteristic of the zwitterionic form of l-lysine. They indicate that the size of polymerized fragments was much smaller than in 1, at best short oligomer chains were formed leaving many unreacted COO$^-$ groups. At the same time strong C-O-C asymmetric stretching peak at 1080 cm$^{-1}$ and a weaker symmetric stretching at 860 cm$^{-1}$ reveal presence of ether groups. Thus, lysine monomers or oligomers in 2-3 are connected by ether bonds.

Mass-spectra measurements confirm polymerization of L-lysine under femtosecond laser exposure: the relative intensity of lysine monomer ion at m/z=145 decreased for 2 and practically disappeared for 1 parallel to decrease of NH$^-$ and COO$^-$ signals, while stronger signal of CN$^-$ and CNO$^-$ attributable
to peptide bonds appeared (Figure 3b). 1 yielded especially strong peptide signals, confirming large scale of polymerization.

Thus, chemical analysis demonstrates difference in structure and formation mechanism of macromolecular products for kilohertz and megahertz laser irradiation regimes (Figure 3c). Kilohertz irradiation with high-energy pulses promotes polymerization and formation polymer clusters. Cross-linking of polymer chains gives these clusters a rigid structure.

![Figure 3](image)

**Figure 3.** (a) ATR FTIR spectra of 1-3. (b) Relative intensity of TOF-SIMS negative ions signals with m/z=15 (NH\(_2\)), 26 (CN\(_{-}\)), 42 (CNO\(_{-}\)), 44 (COO\(_{-}\)) 145 (C\(_6\)H\(_{13}\)N\(_2\)O\(_2\)) for l-lysine, 1 and 2. (c) Proposed structures of L-lysine heavy-weight irradiation products in 1-3.

Partial carbonization produces partly carbonized or even aromatic domains in nanoparticles, while oxidation and reorganization of amide groups forms a shell of polar amide and hydroxyl surface groups. This process is similar to synthesis of carbon nanodots by solvothermal or microwave methods from amino acids [9, 10], and resulting nanoparticles can be described as carbonized polymer dots [8]. Exposure to high repetition nanojoule femtosecond pulses produces flexible macromolecules composed of lysine monomers or short oligomers connected by ether bonds. Curiously, despite this difference in structure both types of products exhibit excitation-dependent visible photoluminescence typical for carbon nanodots. It can be hypothesized that fluorophores are produced by partial carbonization and formation of conjugated structures from sections of the polymer/oligomer chains.

4. Conclusion

We demonstrated that under exposure to femtosecond laser pulses chemical products with visible absorption and photoluminescence are formed from l-lysine aqueous solution independently of the Maillard reaction. Molecular weight, structure and chemical composition and absorption and photoluminescence characteristics of products strongly depend on the irradiation regime. Exposure to high-energy pulses in the laser breakdown regime is especially effective for obtaining carbon
nanodots-like luminescent products. Our results shed new light on possible mechanism of formation of luminescent products in material of living cells and tissues under pulsed laser irradiation.

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