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Photoactivated Green Fluorescence Emission by Femtosecond Oscillator from Indole Solutions

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Abstract We reported a novel femtosecond-laser-activated fluorescence emission from indole solutions upon excitation by the second harmonic wavelength of a femtosecond oscillator. A new absorption band around 400 nm and corresponding fluorescent band in the green domain were produced after the irradiation of femtosecond laser. This femtosecond-laser-activated luminescence process that allows the use of visible wavelength as a substitute for UV light to excite fluorescence from indole would extend applications based on indole chromophore. Furthermore, the photoactived emission can act as a fluorescence lifetime probe to measure the polarity in complex biological systems since it is polarity-sensitive. High performance liquid chromatography with fluorescence detector (HPLC-FLU) and high performance liquid chromatography with mass spectrometer (HPLC-MS) analysis demonstrate that the origin of the photoactivated fluorescence is new molecular species that generated in indole solution upon femtosecond laser irradiation.

Keywords Indole · Femtosecond laser · Photoactived fluorescence · Absorption · Fluorescence lifetime · Chromatohraphy

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Introduction

Mode-locked Ti: sapphire femtosecond laser is a well-known light source in many fields. Compared with continuous wave (CW) and long pulsed lasers, femtosecond (fs) laser has a very prominent feature: participation of various nonlinear processes enabled by high localization of laser photons in both time and spatial domains. Therefore, it is expected that fs laser may induce a different phenomena when it interacts with matter compared with CW and long-pulsed lasers [1]. Various luminescence phenomena such as two-color fluorescence [2], fluorescence enhancement [3], and laser-activated fluorescence [4] induced by fs laser have been intensively investigated in the past two decades triggered by the increasing applications in optical storage, three-dimension display, and microscopy for biological science.

On the other hand, fluorescence of indole is an important subject since the indole chromophore is responsible for most of the UV absorption and fluorescence in proteins and thus is essential in UV-Vis spectroscopic studies on proteins [5–13]. Demonstrated in literature using continuous wave excitation source, the maximum absorption of indole in solvents between UV and visible light range is at about 260 nm and 286 nm [14], and the usual emission spectrum of indole is observed around 340 nm [15]. Relatively, investigations of indole fluorescence excited by using ultrafast laser sources are much less numerous. Recently, Qiao et al. reported the abnormal fluorescence spectra upon excitation of a Ti: Sapphire fs regenerative amplifier. However, other involved nonlinear processes such as multi-photon and optical breakdown complicated the investigation because of high single pulse energy [16].

This study focuses on the luminescence from indole solutions using Ti: Sapphire fs oscillator as the excitation

source, which provided much lower pulse energy, thus complex higher-order nonlinear process could be avoided. Upon irradiation of the second harmonic wavelength of a mode-locked Ti: Sapphire fs oscillator, the indole solution was photoactived, giving rise to an ever increasing green fluorescence emission. A new absorption band and corresponding emission band appears, indicated by absorption and emission spectrum measurement by using spectrophotometer. The novel emission of indole in various solvent systems is also characterized by investigating the in situ femtosecond-laser fluorescence spectrum and time-resolved fluorescence lifetime. High performance liquid chromatography with fluorescence detector (HPLC-FLU) and high performance liquid chromatography with mass spectrometer (HPLC-MS) analysis demonstrate that new species was generated in indole solution upon femtosecond laser irradiation, which is responsible for the new photoactivated fluorescence. Since the luminescence of indole is used intensively when studying biological systems and indole nucleus offers intrinsic fluorescent probes of protein conformation, dynamics, and intermolecular interactions, femtosecond-laser-activated luminescence process opens a promising unique spectral window for fluorescence detection involving the indole.

Material and Methods

All experiments are performed at room temperature. Indole (99% pure; Sigma-Aldrich, America) is dissolved in methanol, ethanol, and ether (HPLC grade, China) as a 5×10^{-2} M concentration for each system.

The experimental setup is shown in Fig. 1. Irradiation of indole solutions were accomplished using a commercial Ti: sapphire oscillator system (Micra, Coherent Inc., America), which is operated at a repetition rate of 84 MHz, provides ~100 fs (FWHM) laser pulses with a central wavelength at 800 nm. In the experiment, the fundamental output from Ti:sapphire laser is collimated and frequency-doubled using an I-type β-barium borate (BBO) crystal. Then the fs pulses with wavelengths of 400 nm was separated from the fundamental wavelength by a barrier filter and focused into the sample located in a $10\times10\times30$ mm^3 quartz cuvette through a 100 mm focal-length lens. The irradiated fs laser power at the wavelength of 400 nm was 16.5 mW and kept throughout the all measurements. The only exception is the power-dependent fluorescence intensity measurement in which three irradiation laser powers (9 mW, 16.5 mW, and 26 mW) were used.

In situ femtosecond-laser fluorescence spectrum measurement was performed by focusing the fluorescence signal that transmits at the right angle into a grating spectrometer (Shamrock 303i, Andor, Ireland) using a collective lens with 50-mm focal length. Fluorescence lifetime is detected by the same perpendicular configuration through a time-resolved system consisting of a sync detector photodiode (TDA200, PicoQuant, Germany), single photon avalanche diode (PDM 50CT, PicoQuant), and a PCI-board for time-correlated single photon counter (Timeharp 200, PicoQuant).

The absorption and emission spectra of indole solution before and after fs laser irradiation were obtained using a commercial UV-Visible-NIR spectrophotometer (JASCO V-570, Japan) and a research-grade fluorescence spectrophotometer (JASCO FP6500, Japan), respectively. In both cases, Xe arc lamps were used as the source. Measurements were carried out within 24 h after samples were irradiated by the fs

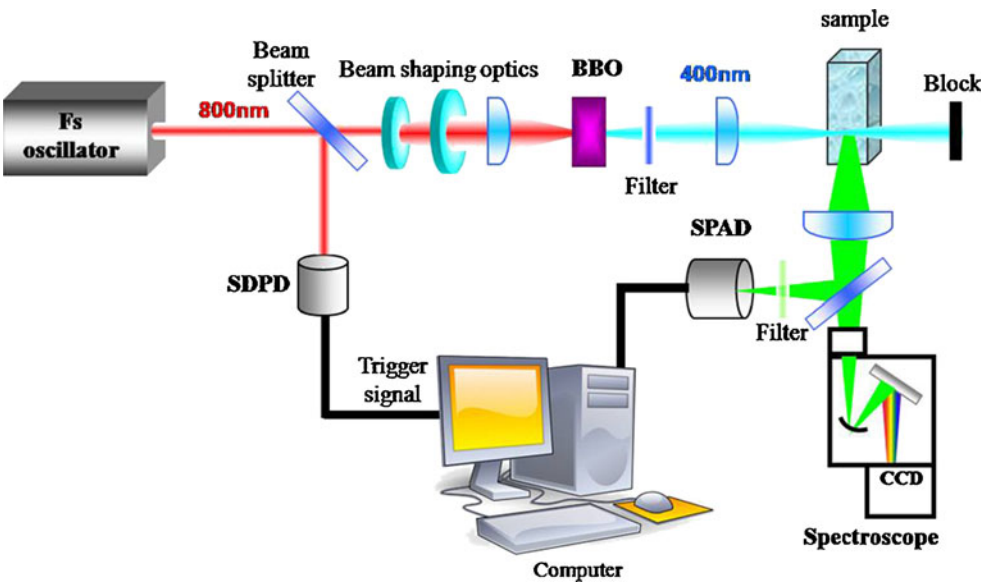

Fig. 1 Schematic diagram of the experimental setup. *SDPD* sync detector photo diode; *SPAD* single photon avalanche diode; *BBO* β-barium borate crystal

laser and the radiation time of samples is 40 min unless specifically noted.

HPLC-FLU was performed on a Venusil MP-C18 column (150 mm×2.1 mm i.d., 5 μm; Agela Technologies Inc., USA) by a Finnigan Surveyor Plus HPLC system (Thermo Fisher, USA) with a Surveyor FL Plus Detector (Thermo Fisher, USA). The column temperature was set at 30 °C and the gradient eluting program was started from 5% B, and changed to 25% B within 2 min, to 45% B within 5 min, to 80% B within 5 min, then changed to 100% B in another 4 min, and at last held at 100% B for 2 min (solvent A: aqueous solution of 0.1% formic acid; solvent B: ACN of 0.1% formic acid). The total flow rate was 0.5 mL/min. The eluant was detected by Surveyor FL Plus Detector at the excitation wavelength of 400 nm and emission wavelength of 520 nm. A volume of 10 μL indole methanol solutions before and after fs laser irradiation were directly injected without dilution.

HPLC-MS were performed on the same Venusil MP-C18 column by an Acquity UPLC system (Waters) equipped with a Micromass Q-TOF Premier Mass Spectrometer (Waters MS Technologies, Manchester, UK). The separation conditions were the same as those of HPLC-FLU. The eluate was directed to the mass spectrometer without splitting. Mass analysis was performed on a Q-TOF mass spectrometer equipped with an ESI source operating in positive ion mode. The desolvation and cone gas rate were set at 600 L/h at a temperature of 350 °C and 50 L/h, respectively. Source temperature was set at 100 °C. The capillary and cone voltages were set at 3,000 and 35 V, respectively. Data were collected in centroid mode from mass-to-charge ratio (*m*/*z*) 50 to 1,000 at scan time of 0.3 s with a lockspray frequency of 30 s.

Mass-spectrum analysis was carried out via Masslynx 4.1 software (Waters MS Technologies, Manchester, UK).

Results and Discussion

Green fluorescence emission could be observed immediately even by the naked eye once the second harmonic wavelength of the fs laser, namely 400 nm, was incident into the indole-methanol solution. In situ femtosecond-laser fluorescence spectrum in Fig. 2 quantitatively shows that the new luminescence produces the major emission peak at the 522 nm, very different from the normal emission at 340 nm of indole. It is established that there is no fluorescence emission from solution by excitation of the fundamental output of fs oscillator, namely 800 nm, in the experiment. Of particular interest is that the green fluorescence became stronger and stronger with the lapse of irradiation time. This trend was clearly demonstrated by the fluorescence spectrum from indole-methanol solution at various irradiation time points, as shown in Fig. 2. Since the indole solution initially did not show any luminescence properties in the green domain before irradiation of fs laser and fluorescence intensity experienced significant growth

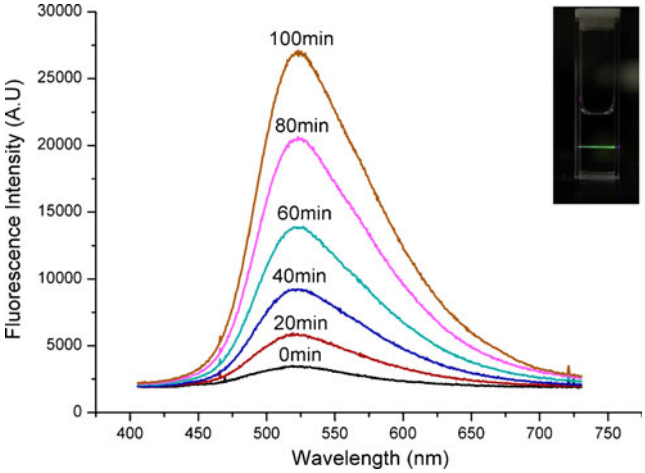

Fig. 2 In situ femtosecond-laser fluorescence spectrum of indole in methanol resulted from the excitation of 400 nm fs laser at the different irradiation times indicated in the figure. *Inset* the experimental picture of fluorescence emission from the indole solution by fs laser irradiation

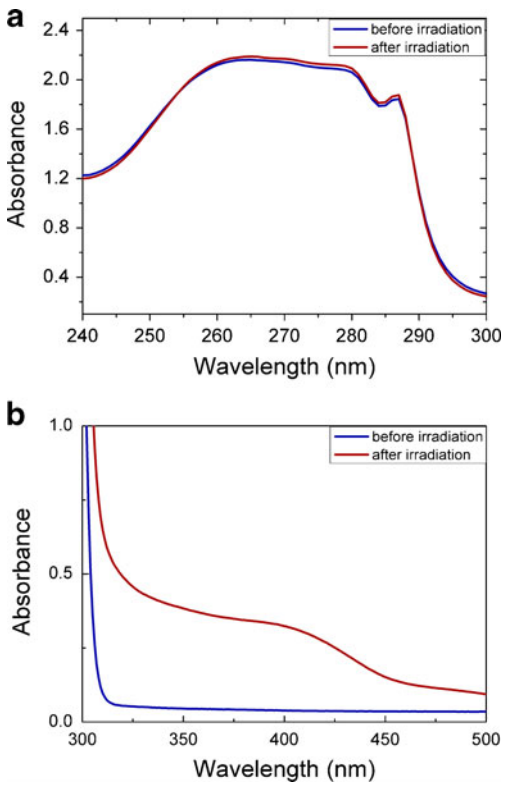

Fig. 3 The absorption spectra of indole-methanol solution before and after fs laser irradiation. **a** Range from 240 nm to 300 nm **b** Range from 300 nm to 500 nm. Concentration of sample is 5×10^{-4} *M* in (**a**) and 5×10^{-2} *M* in (**b**)

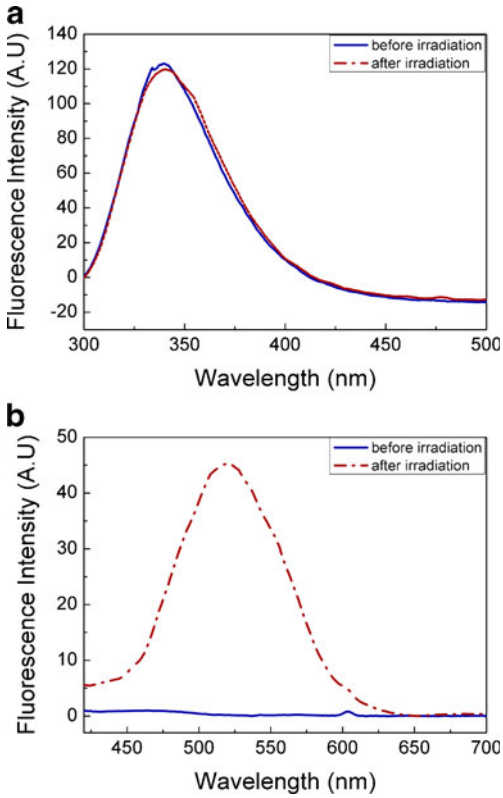

Fig. 4 The fluorescence spectra of indole-methanol solution excited by monochromatic light from Xe arc lamp at wavelengths of **a** 267 nm **b** 400 nm with and without laser irradiation

over irradiation time, this novel emission could be ascribed to a femtosecond-laser-activated luminescence.

More evidence of the photoactived properties termed herein could be found when comparing the absorption and emission spectrum of indole solutions with and without fs

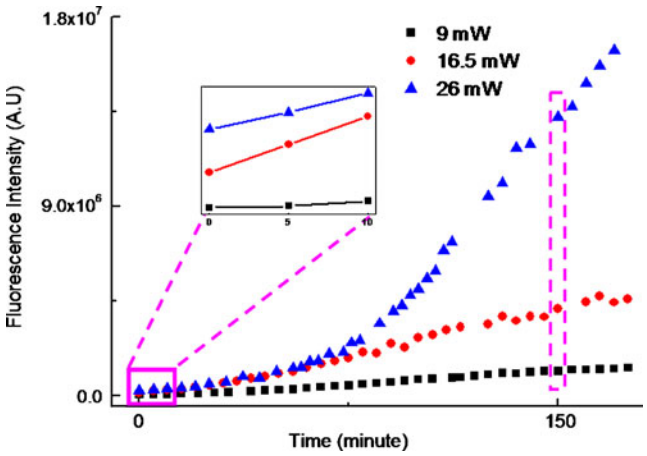

Fig. 5 In situ fluorescence intensity versus the irradiation time at different irradiation powers of 400 nm fs laser **a** 9 mW **b** 16.5 mW **c** 26 mW. Concentration of samples is 5×10^{-2} *M*. *Inset* shows enlargement of the time interval marked with the magenta square in figure

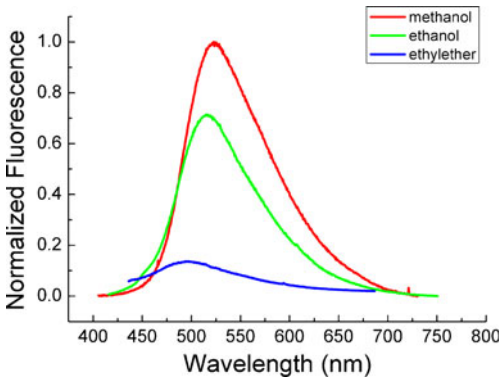

Fig. 6 In situ fs-laser fluorescence spectrum of indole in three different solvent systems (*a*) methanol (*b*) ethanol (*c*) ethylether. Concentration of samples is 5×10^{-2} *M*

laser illumination. Before the irradiation, the spectrum clearly indicated the normal absorption state of indole as indicated in Fig. 3(a). After the irradiation by fs laser, an additional small but obvious shoulder peak appears around 400 nm as displayed in Fig. 3(b), whereas the major part ranging from 240 nm to 300 nm almost remains the same. Figure 4(a) and (b) presented the emission spectra which are excited by 267 nm and 400 nm monochromatic light from a Xe arc lamp, respectively. After photoirradiation, the intrinsic fluorescence peak of indole at 345 nm excited by 267 nm appeared. A new fluorescence peak around 520 nm excited by 400 nm also appeared. These results demonstrated that during the femtosecond-laser-activated luminescence process, fs laser actually play two roles, with one producing a new absorption band (around 400 nm) largely shifting towards longer wavelength by 'activating' the indole solutions, and the other 'exciting' the activated indole solution to give rise to green fluorescence.

On one hand, since monochromic light at 400 nm from Xe arc lamp can produce green fluorescence emission from indole solution irradiated by fs laser, the 'excitation' is

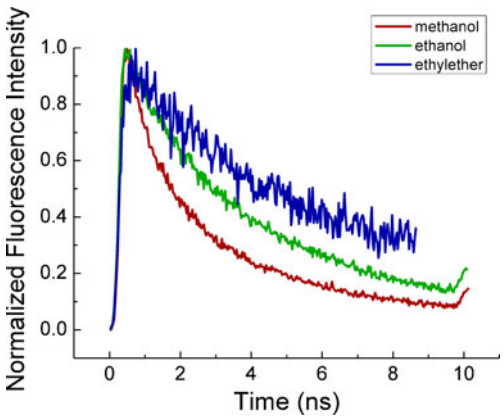

Fig. 7 Time-resolved fluorescence lifetime measurement of indole solved in three different solvents (*a*) methanol (*b*) ethanol (*c*) ethylether

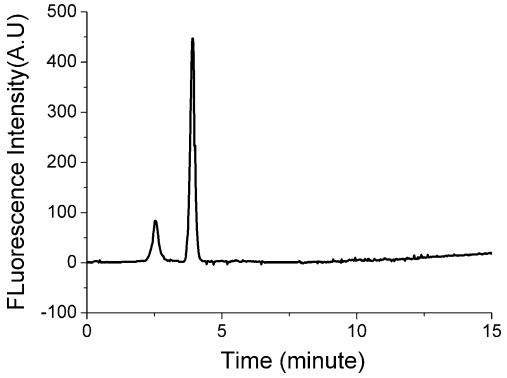

Fig. 8 The HPLC-FLU chromatogram (with background was subtracted) of indole solutions after fs laser irradiation

assumed to be a single photon process. On the other hand, the 'activation' effect may be a complex process. In Fig. 5 the reaction kinetics are characterized by measuring in situ fluorescence intensity as a function of radiation time under three different powers of fs laser which are 9 mW, 16.5 mW and 26 mW, respectively. At different time points, the relationship between the fluorescence intensity and the irradiation power is different. For example, at the beginning of the irradiation, the fluorescence intensity produced by three irradiation powers I was proportional to $\sim I^{1.4}$ (shown in the inset of Fig. 5), whereas at the irradiation time of 150 min, the proportions were changed to $\sim I^{2.1}$. These results indicated that the formation of the green emission cannot be simply ascribed to a single-photon or two-photon process, but to a dynamic one.

Furthermore, it was investigated whether the femtosecond-laser-active process depends on the special solvent. For this purpose, irradiation by fs laser and in situ fluorescence measurement of indole were performed in two other solvent systems, specifically ethanol and ethylether which have very different polarities from methanol. Similar type of fluorescence was observed in both cases. Figure 6 exhibited the fluorescence spectra of indole in methanol, ethanol, and ethylether. Since the dielectric constant of the solvents in order were 32.7, 24.5, and 4.3, respectively [17], covering a range from polarity to almost non-polarity, the results in Fig. 6 show that an increase of the solvent polarity leads to an increase of fluorescence intensity and a shift of the fluorescence band maximum toward longer wavelengths. The lifetime of the photoactivated fluorescence of indole in the mentioned solvents is also characterized. As shown in Fig. 7, the photoactive fluorescence in methanol, ethanol, and ethylether all exhibited single exponential decay, as

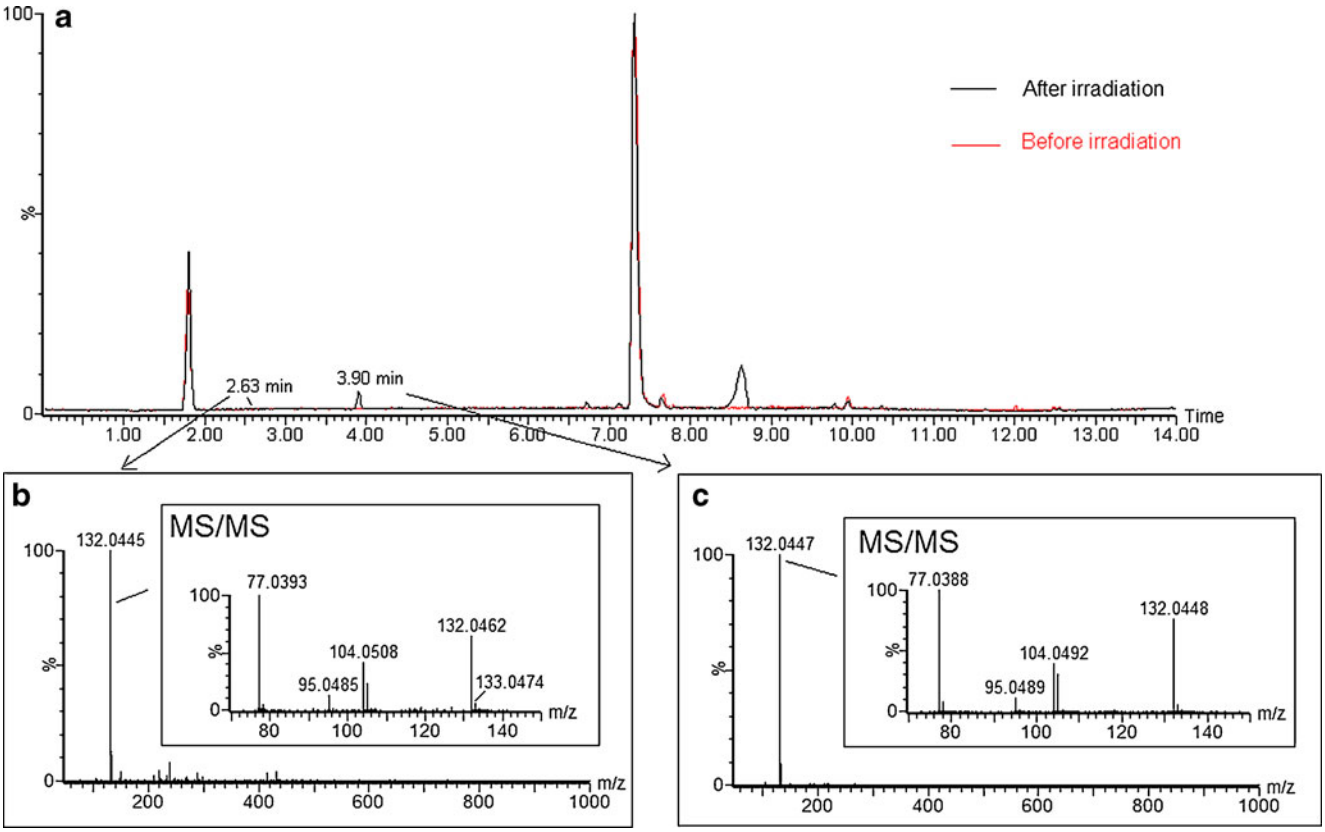

Fig. 9 **a** Comparison of LC-MS chromatograms of indole solutions before and after fs laser irradiation. **b** The MS spectrum of *m/z* 132.0445 at 2.63 min for fs laser irradiated indole solution. *Inset* MS/MS spectrum of *m/z* 132.0445 at 2.63 min. **c** The MS spectrum of *m/z* 132.0447 at 3.90 min for fs laser irradiated indole solution. *Inset* MS/MS spectrum of *m/z* 132.0447 at 3.90 min

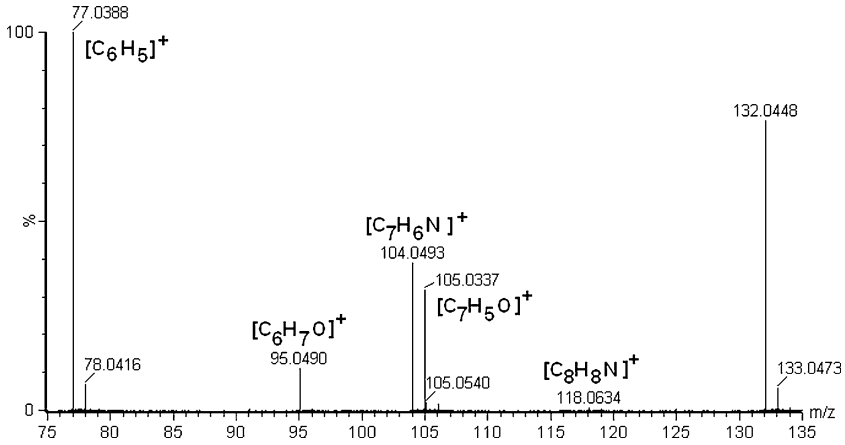

Fig. 10 The elemental composition of the mass peaks in the MS/MS spectrum of *m/z* 132.0447 at 3.90 min in Fig. 9

expected for single emitting species. The lifetime values are 1.45±0.03 ns, 2.55±0.05 ns and 3.38±0.18 ns, respectively, slightly smaller in magnitude than the lifetime of the indole (~4 ns). However, in all cases, the fluorescence lifetime decreases as the polarity of the solvent increases. This trend is very different from the normal condition known from literature in which the lifetime of indole was reported to increase with the solvent polarity [18, 19], manifesting again a completely different fluorescent state.

To find out the origin of this photoactivated fluorescence, we furthermore performed HPLC-FLU and HPLC-MS analysis of the indole solutions. The HPLC-FLU chromatogram and the overlay HPLC-MS chromatogram of indole solutions after fs laser irradiation are shown in Figs. 8 and 9. It can be seen from these figures that the peaks responsible for fluorescence emission in Fig. 8 corresponds to the peaks at 2.63 min and 3.90 min in Fig. 9, which is obviously new molecular species generated after fs laser irradiation compared with the case without laser irradiation. Mass spectra for the peaks at 2.63 min (Fig. 9(b)) and 3.90 min (Fig. 9(c)) reveal that mass-to-charge ratio in $[M+H]^+$ form of the photoproduced molecules are *m/z* 132.0445 and *m/z* 132.0447, respectively. Then we speculate that elemental composition of the new species based on indole is $[C_8H_6NO]^+$ ($[M+H]^+$), which must have been generated continuously upon the illumination of femtosecond laser, and in turn contribute to the green fluorescence emission. Since the fluorescence species are always characterized by a certain conjugated system containing π electrons, several possible molecular structures could be speculated on the basis of indole structure and the MS/MS spectra of $[C_8H_6NO]^+$, such as benzoyl cyanide, 2-cyanobenzaldehyde and 1-oxoisoindole. However, results of HPLC-MS measurement (data not shown) demonstrate that the molecular species generated upon irradiation can't be benzoyl cyanide and 2-cyanobenzaldehyde. Detailed mass-spectrum analysis of the elemental composition of the species at the retention time of 3.90 min, shown in Fig. 10, exhibited that peaks at *m/z* 104.0493 and *m/z* 105.0337 are product ions, resulting from the elimination of a CO group and a CNH group from the parent ion $[C_8H_6NO]^+$, respectively. Based on the study of previous literature [20], in which 2,3-dihydro-3-hydroxy-l-oxoisoindol containing an l-oxoisoindol group produced ion peaks at the *m/z* 104 and *m/z* 105 with similar intensities in electron ionization mass spectrometry (EI-MS) measurement, we propose the following picture. In the structure of 1-oxoisoindole molecule, the 1C-2N bond is relatively weak and very likely to be broken under collision-induced dissociation (CID) mode, and thus both the CO group and the CNH group are possible to be eliminated from the parent molecule to form $[C_7H_6N]^+$ ion and $[C_7H_5O]^+$ ion, respectively. Therefore, we tend to consider 1-oxoisoindole be a possible candidate and its molecular structure shown in Fig. 11.

Conclusion

After the irradiation of fs oscillator, absorption and emission spectra of indole was changed evidently. A new absorption band at 400 nm and corresponding fluorescence emission in the green domain were produced. The activated

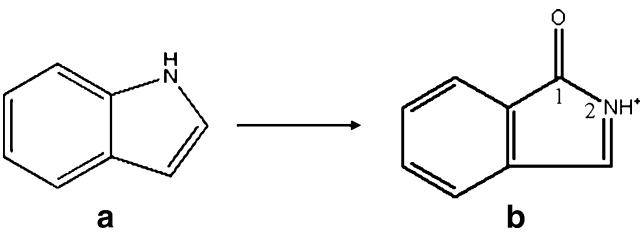

Fig. 11 A speculated molecular structures that may generated upon the irradiation of fs laser and responsible for green fluorescence emission. Molecular structure of **a** indole; **b** 1-oxoisoindole

fluorescence from indole in three different solvents was measured with different polarity and characterized by fluorescence lifetime measurement. It is known that indole chromophore offers intrinsic fluorescent probes of protein conformation, dynamics, and intermolecular interactions, and its luminescence is used to study complex biological systems [21, 22] excited by the 280 nm wavelength. Clearly, irradiation by fs laser at 400 nm 'activates' fluorescent emission different from the normal one. The use of visible wavelength as a substitute for UV light to excite fluorescence of indole chromophore can have numerous advantages such as absorption avoidance of DNA, reduced phototoxicity, deeper penetration, and longer observation time. These open prospects for advanced photonic application, particularly in biomedical imaging. The polarity-sensitive lifetime of the photoactived emission from indole could act as a probe for micropolarity measurements of biological systems. HPLC-FLU and HPLC-MS analysis showed that new molecular species was generated in indole solution upon femtosecond laser irradiation, which is responsible for the new photoactivated fluorescence. To further investigate the underlying mechanism of this photoactive process, more measurements involving physics and stoichiometric structure, especially nuclear magnetic resonance (NMR) should be carried out in the future.

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