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ORIGINAL PAPER

Nonlinear Excitation of Tryptophan Emission Enhanced by Silver Nanoparticles

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Abstract We measured and analyzed the behavior of the fluorescence of tryptophan water solutions with and without silver nanoparticles, excited by one, two and three photon processes. Two different colloids with silver nanoparticles with distinct diameters (0.65 nm and 9 nm) were used in the experiments. Fluorescence quenching was observed with one and two photon excitation. However, upon three-photon excitation, significant fluorescence enhancement was observed in the colloid. In this case excitation of the amino acid is assisted by the nonlinear absorption of infrared light by the silver nanoparticles. In this paper we are proposing a new way to explore metallic nanoparticles to enhance autofluorescence of biomolecules.

Keywords Tryptophan · Autofluorescence · Metallic nanoparticles · Nonlinear excitation

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Introduction

The nonlinear optical properties of organic materials have been subject to extensive studies aimed at understanding their intrinsic origin and also at the possible use of these systems in photonic applications, such as microscopy. In particular, tryptophan ($C_{11}H_{12}N_2O_2$) is an important biomolecule. For many organisms including humans, it is one of the essential amino acids (building blocks of proteins), which cannot be synthesized by the by the human body and therefore must be part of its diet. Tryptophan is a precursor for serotonin, one of the key brain chemicals involved in mood regulation [1]. It is also necessary for the production of niacin, a water-soluble vitamin whose derivatives such as NADH play essential roles in energy metabolism in the living cell [2]. Furthermore, tryptophan is involved in the body's regulation of sleep. It has also been found that people suffering from migraine headaches have abnormal levels of tryptophan, and its monitoring and control may be helpful. Tryptophan is a fluorescent molecule which has been subject of a recent detailed study [3] aiming at providing a new insight into the interpretation of the fluorescence origin. Most of the intrinsic fluorescence emission of a folded protein is due to excitation of tryptophan residues, with additional contributions by tyrosine and phenylalanine. In addition, it is a relatively rare aminoacid, therefore many proteins contain only one or a few tryptophan residues. Thus, tryptophan fluorescence analysis is a very sensitive diagnostic method for the conformational state of proteins [4]. Moreover, its autofluorescence can be explored in optical microscopy. Recently, nonlinear cellular optical microscopy was demonstrated using tryptophan as the autofluorescent molecule [5]. Multi-photon fluorescence microscopy has important advantages over conventional epifluorescence or confocal

microscopy, especially for the imaging of thick biological specimens. The most important of these is that photobleaching occurs only in the immediate focal region, rather than over the complete volume as in confocal fluorescence. In nonlinear microscopy usually an infrared source is used, also resulting in less scattering and greater penetration through thick tissue. The low phototoxicity produced by two and three-photon imaging compared to wide-field or confocal imaging offers exciting prospects for long-term *in vivo* imaging. Moreover, two and three-photon absorption can be used to excite levels corresponding to wavelengths deep into the UV, without the necessity for UV optics. Three-photon excitation has been predicted to yield greater resolution and deeper excitation into the UV absorption levels than two-photon excitation using the same excitation wavelength.

Fluorescence emission of molecules can be modified by their interaction with metallic nanoparticles (NP) [6]. The linear optical properties of metal NP–molecules systems have been widely explored [7–10]. In particular, metal nanoparticles (NPs) do not fluoresce, but rather they can enhance the emission of fluorophores, through local field effects [6, 10]. Metallic nanoparticles scatter light elastically with remarkable efficiency because of a collective resonance of the conduction electrons in the metal (i.e., the localized plasmon resonance). Energy transfer is also observed in systems consisting of metal nanoparticles and organic dye molecules, leading to fluorescence quenching caused by an increased nonradiative rate and decreased radiative rate [7]. Both radiative and nonradiative rates depend on the nanoparticle's size and shape, its distance to the fluorescent molecule and the overlap of the molecule's emission with the nanoparticle's absorption spectrum. One can control the radiative rate of fluorescent molecules by controlling the interaction with metallic NPs.

In this work, we have measured and analyzed the behavior of the fluorescence of tryptophan water solutions with and without silver nanoparticles, excited by one, two and three photon absorption processes. The variation of the colloid emission with NP size and concentration were observed and explained. Our result suggests that three photon absorption process assisted by metallic NPs can be explored as a new tool in fluorescence microscopy. Moreover in this paper we are proposing a new way to explore metallic nanoparticles to enhance autofluorescence of biomolecules.

Methodology

The tryptophan used in our experiments was obtained from the Ajinomoto, Inc. All measurements were performed using water solution of the amino acid at a concentration of 0.1 μM and pH=6±1. The amino acid absorption spectra are characterized by absorption bands centered at 275 nm, which is a contribution from the aromatic side chains of the molecule [11]. Colloids suspensions were added to the aminoacid solution. Two different colloids with silver nanoparticles with distinct diameters were used in the experiments. Silver spheres, with average diameter of 0.65 nm, were purchased from Purest Colloids Inc. (Mesosilver®). Colloids with larger diameter (9 nm) were prepared based on the procedure described by Lee and Meisel [12].

Silver nanoparticles were added to the amino acid solution and its UV–visible absorption spectra were obtained over the 200–700 nm range using a model DV-Z500 spectrophotometer (Beckman, USA).

The luminescence spectra of the colloid were obtained using three different light sources: a Ti–sapphire laser delivering pulses at 76 MHz, with 150 fs pulse duration and 500 mW average power at 800 nm, the second harmonic of a Q-switched Nd:YAG laser (with 8 ns pulse duration at 532 nm) and a UV (180–375 nm) lamp. The fluorescence emission obtained by the UV lamp excitation was measured using a model DV-Z500 spectrophotometer (Beckman, USA). For both laser excitation setups the light beam was focused into the sample with a 10 cm focal length lens (L1). The emitted light was collected along a direction perpendicular to the incident beam and optical filters (F) were used remove the pump scattered light. The fluorescence was sent to a monochromator attached to a GaAs photomultiplier tube and lock-in amplifier (SR530 Stamford Research Dual Phase), as shown in Fig. 1. The lock-in amplifier was triggered by an electrical signal obtained from the laser control electronics. All experiments were performed with the samples contained in a 10 mm long quartz cuvette at room temperature.

Results and discussion

As 9 nm silver nanoparticles are added to the amino acid solution, a peak centered at 400 nm appears in the tryptophan–Ag colloid absorption spectra. Figure 2 shows the linear absorption spectra for different silver NP filling

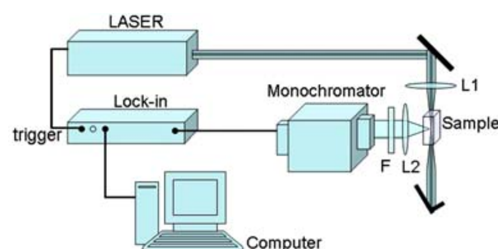

Fig. 1 Experimental setup for nonlinear spectroscopy

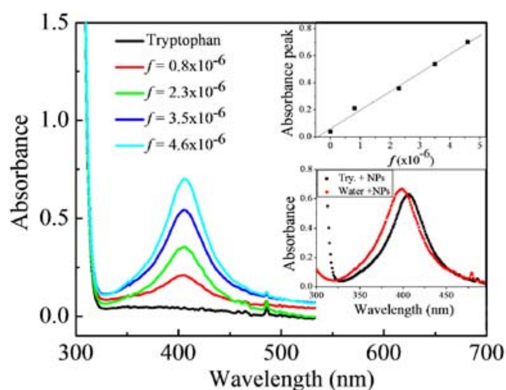

Fig. 2 Absorption spectra for different 9 nm silver NP filling factors in tryptophan solution. *Upper inset*, absorbance peak value for different NP filling factor. *Lower inset*, plasmon band spectra of 9 nm silver spheres in pure water and in the tryptophan solution

factors, *f*, in tryptophan solution. The volume filling factor *f* is defined as the ratio of the total volume occupied by the nanoparticles divided by the total volume of the solution. The upper inset of Fig. 2 shows that the absorbance peak value (at 400 nm) increases linearly with the NP filling factor. It is known that aggregation of tryptophan to metal clusters can lead to remarkably changes of the optical absorption as compared to the bare biomolecule [13]. Moreover, NP–molecules interaction can also lead to displacement of the plasmon peak and to the narrowing of the plasmon bandwidth [14]. The lower inset of Fig. 2 shows the plasmon band spectra of 9 nm silver spheres in pure water and in the tryptophan solution. A 6 nm displacement and bandwidth reduction of 4 nm indicates that some adsorption of molecules on the surface of the metal spheres maybe taken place.

The tryptophan molecules also impose changes on the absorption spectrum of the 0.65 nm silver colloid moving the plasmon absorption peak from 398 nm to 405 nm. Plasmon's bandwidth is also modified, reducing from 100 nm to 95 nm.

The fluorescence spectrum of the tryptophan solution is characterized by a broad band (70 nm) emission with its peak at around 360 nm. This is observed for all excitation light source used on the experiment. The fluorescence spectrum of tryptophan water solution are presented as the black curve on Fig. 3a, b and c. Figure 3a, b, and c refers to the fluorescence spectra behavior of the solution with different silver 9 nm NP concentration, excited respectively with the UV lamp, Nd:YAG laser and Ti:sapphire laser.

In Fig. 3a the fluorescence spectra of the colloids pumped with one photon at 270 nm are presented. One can observe a quenching in the amino acid emission with the increasing of 9 nm NP concentration. Moreover, fluorescence of the tryptophan can be partially absorbed by the plasmon band (at 400 nm) creating a valley on the emission spectrum. The tryptophan fluorescence is governed by the magnitudes of the radiative rate, Γ, and the sum of the nonradiative decay rates, κ_{nr}. The lifetime of a fluorophore, τ, is given by the inverse of the total decay rates, $(\Gamma+\kappa_{nr})^{-1}$. The quenching process can be understood by damping of the dipole oscillators by the nearby metal particles [6, 15]. Moreover metal–molecule interaction can lead changes on the quantum yield and lifetime of the fluorescent molecule, expressed by an increase in the radiative and nonradiative rates of the system [6]. Similar quenching is also observed when the colloid is excited by a two photon light absorption process (Fig. 3b). The inset on Fig. 3b shows that the fluorescence's intensity of the tryptophan solution has a square dependence with the laser pump intensity (at 532 nm), consistent with simultaneous

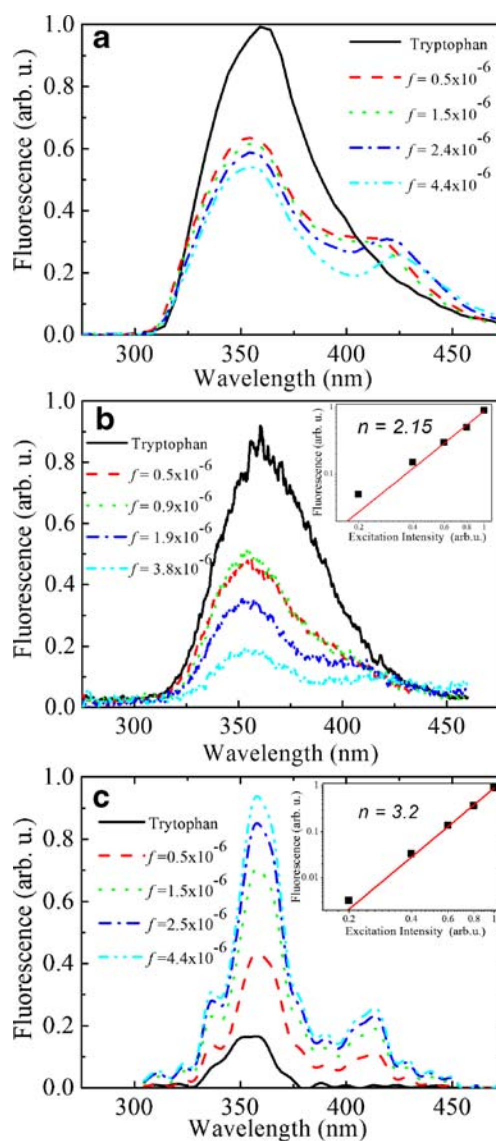

Fig. 3 Fluorescence spectra of tryptophan solution with different 9 nm silver NP filling factors excited by (**a**) one, (**b**) two, and (**c**) three photon absorption process. The *insets* on **b** and **c** show the fluorescence peak intensity as function of the excitation intensity

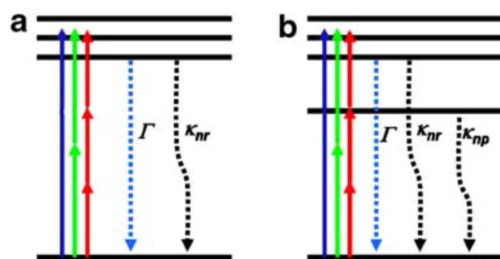

Fig. 4 The excitation and the relaxation processes of **a** the tryptophan solution and **b** the tryptophan–9 nm Ag colloid

absorption of two photons. Excitation and the relaxation (radiative and non radiative) processes of the tryptophan solution and the colloids are represented in Fig. 4. The new relaxation pathways introduced by the metal nanoparticles (nonradiative decay rate, κ_{np}) are shown in Fig. 4b. Although, one photon at 270 nm and two photons at 532 nm are resonant with the excited states of the molecule, these wavelengths are not in resonance with the plasmon energy level.

In Fig. 3c the fluorescence spectra of the colloids pumped with three photons at 800 nm are presented. The excitation of the solution with the 800 nm laser requires a simultaneous absorption of three photons. This is verified on the inset of Fig. 3c, that shows a cubic dependence of the tryptophan's emission intensity with the intensity of the 800 nm pump beam. In that case, the fluorescence emission is enhanced with the addition of NPs into the solution. We believe that the presence of nanoparticles introduces a new energy level in the solution which matches with the energy of two photons at 800 nm (Fig. 4b), providing an enhancement for absorption of a third photon by the amino acid. The emission spectra obtained with the IR excitation were partially disrupted by a dichroic mirror used to eliminate IR scattered light. Even so, fluorescence enhancement was clearly observed with three photon excitation. The time-resolved fluorescence analysis of the amino acid solution was performed using a time-gated camera (La Vision Picostar HR). The time dependence of the solutions' emissions is presented in a semilogarithmic plot in Fig. 5. When excited by the 800 nm laser the tryptophan presents a decay time of approximately 4 ns (circles in Fig. 5). Moreover, the emission of the tryptophan–9 nm silver colloid presents a fast pulse emission, at $t=0$, followed by a slow exponential decay (squares in Fig. 5). The fast pulse peak cannot be resolved by our system resolution (200 ps) and it can be due to a surface Raman process [7]. The temporal analysis of the slow emission of the samples shows that the lifetime of the excited state of the tryptophan does not decrease with the presence of NPs in the solution, corroborating to the fact that no quenching is observed with the IR excitation.

The resonant absorption of two-photon by the NPs is related to the imaginary part of the effective third-order optical susceptibility, $\chi^{(3)}$, that increases with the addition of NP. The dependence of $\chi^{(3)}$ of the colloid with the silver NPs concentration can be described through the generalized Maxwell–Garnett model [16]. Accordingly, the silver NPs, with dielectric constant $\varepsilon_{\mathrm{NP}}$, are assumed to be spheres (smaller than the characteristic distance between the NPs) embedded in a host having dielectric constant ε_{h}. The suspension is considered to be macroscopically isotropic, and from the local field theory [16], the effective nonlinear susceptibility for colloids with small filling fraction can be written as

$$\chi_{\mathrm{eff}}^{(3)} = f\left|\frac{3\varepsilon_{\mathrm{h}}}{\varepsilon_{\mathrm{NP}} + 2\varepsilon_{\mathrm{h}}}\right|^2 \left[\frac{3\varepsilon_{\mathrm{h}}}{\varepsilon_{\mathrm{NP}} + 2\varepsilon_{\mathrm{h}}}\right]^2 \chi_{\mathrm{NP}}^{(3)} + \chi_{\mathrm{h}}^{(3)} \quad (1)$$

Where $\chi_{\mathrm{NP}}^{(3)}$ and $\chi_{\mathrm{h}}^{(3)}$ are the diagonal third-order optical susceptibilities of NPs and host, respectively. From Eq. (1) one can expect the two-photon absorption of 800 nm by the colloid to increase with the addition of NPs into the solution.

To confirm whether the excitation of the plasmon energy state is the responsible for the fluorescence enhancement of the tryptophan–silver colloid, we pumped the sample simultaneously with the second harmonic (532 nm) of the Nd:YAG laser (ULTRA CFR/Big Sky Laser Technologies) and with a dye laser (Coumarine) operating at 400 nm,

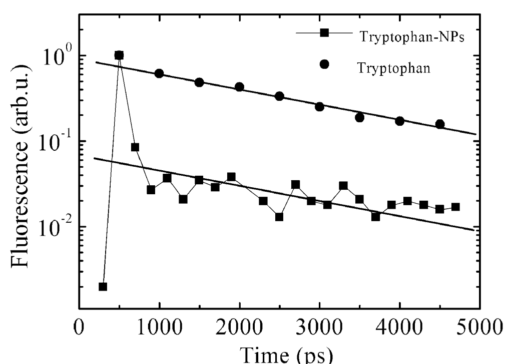

Fig. 5 Fluorescence lifetime of tryptophan and tryptophan–NPs suspension. The *solid lines* are "guides to the eyes"

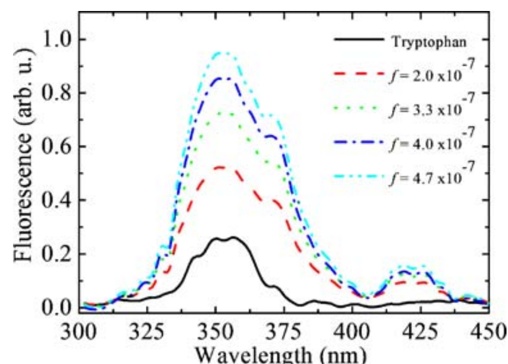

Fig. 6 Fluorescence spectrum of tryptophan solution with different silver subNP filling factors excited by three photon absorption process

resonant with the Ag plasmon level. The dye laser (linear resonator without tuning elements) was pumped by the third harmonic (355 nm) of the same Nd:YAG laser. The delay time between the green (532 nm) and blue (400 nm) pulses were reduced by compensating their optical pathway. A 2±1 ns delay time (shorter than the tryptophan lifetime) was measure at the sample. With the double laser pump, an increase of the colloid fluorescence was observed, compared to the emission of the sample excited only with the green laser. Moreover, no fluorescence was observed exciting the colloid with only blue light.

Finally, it should be mentioned that the dependence of the effective $\chi^{(3)}$ with the NPs size is not explicitly present in Eq. (1). However it is known that the value of $\chi^{(3)}_{NP}$ increases by decreasing the NPs radius [17]. Therefore an increase on the fluorescence emission of tryptophan–Ag colloid would be expected if smaller particles were used. In Fig. 6 the behavior of the fluorescent spectrum of the tryptophan–colloid with different concentration of 0.65 nm diameter NPs is shown. In that case, enhancement of the fluorescence emission is also observed with the addition of the metal particles. Moreover, the concentration of 0.65 nm NPs in the tryptophan solution needed to elicit the same fluorescence enhancement in the colloid as the 9 nm Ag NPs was one order of magnitude lower.

Conclusions

In this work we have demonstrated the possibility of using nano- and subnano-sized silver particles as a new tool to enhance autofluorescence of biomaterials. The excitation of tryptophan was assisted by silver particles' two-photon absorption. The nonlinear absorption process is a function of the colloid filling factor, as described by the generalized Maxwell–Garnett model. We also showed that significant enhancement of the colloid emission can be obtained with small concentrations of subnano silver particles. Fluorescence quenching was observed with one and two photon excitation. The three photon absorption process assisted by NPs can be explored as a new tool in fluorescence microscopy and possibly diagnostic procedures.

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