

PHOTO-EXCITATION NATURE OF AROMATIC AMINO ACIDS UNDER ELECTRIC FIELD: A FLUORESCENCE SPECTROSCOPY STUDY

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Abstract. This study demonstrates the effect of the external electric field during the real-time photoexcitation process in tryptophan and tyrosine under the steady-state fluorescence. The electric field was applied from 1.0 to 12 V/cm during photoexcitation by xenon flash lamp. The tryptophan intensity increased gradually and reached double at 6 V/cm, and at higher voltages, up to 12 V/cm, the intensity decreased with redshift (357 to 363 nm). In the case of tyrosine, the intensity was decreased with increasing voltage. There was no change in $\lambda_{\max}$ in UV absorption spectra. The interesting observation was a two-fold increase in the quantum yield of tryptophan. The obtained results are promising and new, and would be useful in improving fluorescence technology in molecular imaging and characterization of fluorophores.

Key words: Fluorescence spectroscopy, tryptophan, tyrosine, electric field.

INTRODUCTION

The use of fluorescence as an analytical technique has been growing in the last 30-35 years. It is one of the most important and extensively applied methods in biophysics, biochemistry, molecular biology, and biotechnology research. This technique has drawn the attention of many researchers due to quantitative understanding in the biological systems like genetic analysis, protein characterization, DNA sequencing, live-cell imaging, medical diagnostic, etc.

The electric field has been used in biology and medicine in various applications. It can regulate various cell functions like cell growth, adhesion, reorganization of cytoskeleton, contractility, differentiation, proliferation, protein

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secretion and gene expression [14]. Maria Hilczer has analysed the effect of external electric field on fluorescence quenching due to electron transfer from a photoexcitation donor to an acceptor theoretically [8]. Mehata *et al.* studied the external field effect on absorption spectra of 6-hydroxyquinoline (HQ) deoppedin polymer film of poly(methyl methacrylate) or poly(vinyl alcohol) [12]. It has been observed that HQ is affected by electric field in the polymer matrix due to permanent dipole moment and molecular polarizability in ground states. Chen Ruiyun *et al.* hypothesized that the electron transferred between a single molecule and electron acceptor in the polar poly(methyl I methacrylate) (PMMA) matrix contributes the diverse response of fluorescence intensity to the electric field [3]. Further, their observation showed that the local environment of polymers plays a significant role in fluorescence intensity response to electric fields. In steady-state spectrum, dual emission was observed from the excited states of normal (N^*) and tautomer (T^*) form reported in 4 N,N (dimethylamino)-3 hydroxyflavon (DMHF) in PMMA film [6].

Among the three fluorescent amino acids, tryptophan (Trp) is a powerful fluorescent probe used in protein research, which can be applied for the study of folding/unfolding, substrate binding, quencher accessibility etc. The λ_{max} of Trp is quite sensitive to its local environment, ranging from 308 to 355 nm, and roughly correlates with the chromophore's degree of solvent exposure. Trp is the most abundant and is present at concentration of about 1 % soluble cytoplasmic proteins, and up to 3 % in membrane proteins [9]. Although tyrosine (Tyr) has a quantum yield similar to Trp, the indole group of Trp is considered the dominant source of UV absorbance at 280 nm and emission at 350 nm in proteins [2, 5].

In the present investigation, efforts have been made to understand whether the external electric fields (EEF) interfere with the photo-excitation process during excitation of natural fluorophores tryptophan and tyrosine. The experimental data have been systematically analysed using standard fluorescence tools, and the possible mechanism has been exploited.

MATERIALS AND METHODS

MATERIALS

L-tryptophan was obtained from SRL, L-tyrosine from Loba Chemie, India. All chemicals were analytical grade and used without purification. Double-distilled water was used throughout the study for experimentation of samples (pH-7.0).

UV VISIBLE SPECTROSCOPY

UV absorption of the amino acids was measured on NanoPhotometer spectrophotometer (Implen, USA), in the wavelength range of 200–400 nm. The quartz cuvette with 10 mm path was used for measurements.

FLUORESCENCE SPECTROSCOPY

The intrinsic fluorescence measurements of amino acids were recorded on Cary Eclipse spectrofluorimeter (Varian, USA) at the excitation wavelength of tryptophan (279 nm) and tyrosine (274 nm). The PMT voltage was 450 V for tryptophan and 520 V for tyrosine. The excitation and emission slit width were 10 nm for all measurements. The path length of 10 mm fluorescence quartz cuvette was used.

L-tryptophan was used as a standard for calculating the quantum yield. The quantum yield (Φ) was determined using the following equation:

$$\Phi_u = \frac{A_s F_u n_u^2}{A_u F_s n_s^2} \times \Phi_s \quad (1)$$

where A_s and A_u are the absorbance values of the standard and unknown sample, respectively; F_s and F_u are the area under the curve of the fluorescence spectra of standard and the unknown sample; n ($n_s = n_u$) is the refractive index of the solvent; Φ_s, Φ_u are the quantum yield of the tryptophan standard and unknown sample [7].

ELECTRODE DESIGN AND EXPERIMENTAL SETUP

Fixed concentration of Trp (0.016 mg/mL) and Tyr (0.5 mg/mL) were dissolved in distilled water. Further, three milliliters of Trp and Tyr were taken in quartz cuvette for the study. A typical arrangement was made for the observation of the photo-excitation phenomenon of amino acids under the external electric field i.e., the field was applied to the fluorophore. At the same time, the samples were excited. For this purpose, steel electrodes were designed as shown in Fig. 1. The electrodes were immersed in the quartz cuvette with a specific alignment and position. The electrodes were maintained at fixed distance and correctly angle throughout that experiment, so that they would not interfere with the excitation and emission beam of light (Fig. 1). Amino acid solutions were placed in the quartz cuvette, and a DC power supply was used for the experiment. The milliammeter was connected to measure the electric current passed through the samples. In this study, the samples were excited by a xenon lamp during the process of fluorophore

photo-excitation at room temperature, and real-time changes in the photophysical process of both amino acids in solutions were measured.

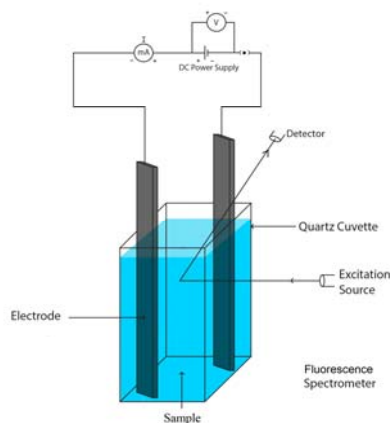


Fig. 1 Schematic representation of the cuvette with electrodes immersed in sample. DC voltage was applied during photo-excitation process.

RESULTS AND DISCUSSION

Trp has a small dipole moment, but when excited in UV wavelength, it creates a large static dipole at an energy level that is very sensitive to the extent of the water molecules solvation [1]. Most of the literature data showed that the effect of the external electric field can bring significant changes in molecular energy, bond length, charge distribution and dipole moments of biomolecule [10, 13]. The fixed concentration of Trp (0.016 mg/mL) and Tyr (0.5 mg/mL) were subjected to varied amplitudes of external electric field (0.0 to 15.0 V/cm) and showed a significant spectral shift at all applied voltages. Fig. 2a shows the progressive increase in Trp fluorescence intensity upon application of voltage, resulting in a significant enhancement by ~ 2 fold as compared to the control Trp. The maximum intensity enhancement was noticed at 6.0 V/cm, further, the intensity was gradually reduced at higher V/cm (Fig. 2a and 2b). Conversely, Tyr intensity decreased with increasing EEF (Fig. 2d). The fluorescence enhancement possesses a direct relation with the quantum yield of a fluorophore. Therefore, to determine the amount of fluorescence enhancement of Trp, the quantum yield of Trp as a function of EEF is plotted in Fig. 2c. It was reported that the increase in quantum yield suggests the decrease in the non-radiative decay in the polar solvent. Therefore, it appears reasonable to indicate that the changes in quantum yield was associated with the

change in the non-radiative decay rates (k_{nr}) [9]. Generally, Trp indole ring has a permanent dipole moment pointing from N1 in the five-membered ring to C5 in the six-membered ring. Therefore, the larger the aromatic ring of Trp, the more accessibility to π - π and/or cation- π interactions. Thus, in the presence of EEF, it could be possible to emphasize that EEF may increase the fluorescent sensitivity and accessibility of the charge densities of the amino acids. Józef Mazurkiewicz *et al.* studied the effect of external electric field (EEF) of MV/cm upon tripeptide inner salts, which was simulated involving HyperChem 8.0 software (theoretical experiment). The result showed that EEF caused the changes in molecular energy, bond length, dipole moment, charge distribution, and orientation along the field. Further, they showed that EEF could induce an increase in the positive charge density on the hydrogen atoms of the N^+H_3 , peptide bond NH, NH_2 , and COOH groups and a decrease in the negative charge density on the oxygen atoms of the peptide bond carbonyl groups. These changes were seen when the external electric field (MV/cm) has been optimized to bring change in charge distribution, potential, and dipole moment for tripeptides, which agrees with our optimized applied field (V/cm). In contrast, they did not mention simulation for aqueous medium. In our case, we performed *in vitro* experiments on free amino acids dissolved in double distilled water (pH = 7.0), and we applied EEF during excitation of amino acids. Therefore, their way of principle is different, hence there may be difference in voltages [10]. Our study showed that the use of higher V/cm resulted into drastic decrease in the fluorescence intensity, and this could be due to denaturation of amino acids. Another simulation study was conducted by Józef Mazurkiewicz and Piotr Tomasik who showed the effect of EEF on proteogenic amino acids, which were divided in two groups [11]. In group I (Ala, Gly, Ile, Leu, Lys, Met, Phe, Pro, and Thr), EEF induced an increase in negative charges whereas in group II (Cys, Ser, Tyr, and Val), EEF instigated the opposite effect. They concluded that an increase in EEF strength reduces molecular energy and increases dipole moment in non-ionized amino acids.

Further, the effect of EEF on Trp displayed a remarkable gradual redshift (λ_{max}) from 357 to 363 nm up to 6.0 V/cm, and this shift seems to remain unchanged at high EEF as presented in Fig. 3a and in the shifted spectrum shown in Fig. 3b and 3c. This indicates the increase in the polarizability (Δf) of Trp molecules and solvent. At high EEF, we observed a stable redshift around ~363 nm for all observations. Based on observed spectral shift, it can be concluded that EEF induced the changes in emission energy and surface charge distribution of Trp molecules. It is also possible that these molecular changes occurred in Trp could be able to enhance the fluorescence intensity of the molecules. The electronic distribution in the ground and excited state is different for different fluorophores. Therefore, if such electronic states interfered by an external field that will probably change the charge distribution to some extent and could lead to exhibit some real-time behavioral changes in the intrinsic property of the fluorophore. Hung-Chu Chiang and Nobuhiro Ohta have shown that the field-induced enhancement of

pyrene monomer and excimer fluorescence originates from the field-induced deceleration of the non-radiative decay rate at the emitting state of the excimer fluorescence [4]. Takakazu *et al.* investigated the effect of EEF on green fluorescence protein in a solid material PMMA film, showing that the fluorescence enhancement occurred due to deceleration of non-radiative process [13]. Moreover, the decrease in fluorescence intensity occurring at high voltage is presumed to be due to conformational changes in Trp molecules that reduced the ability of Trp to carry out changes. Generally, the energy of the non-ionized forms of amino acids is more electronegative compared to zwitterions state. However, in the present investigation, we showed that EEF could induce fluorescence enhancement and quenching like phenomena. In contrast to the Trp, the Tyr does not show any fluorescence enhancement under EEF, despite Tyr intensity strongly reduced by applying of electric field. As we have mentioned previously, the reason behind the enhancement is largely due to change in dipole moment and other properties, as Tyr does not have permanent dipole moment and therefore are weakly affected than the Trp.

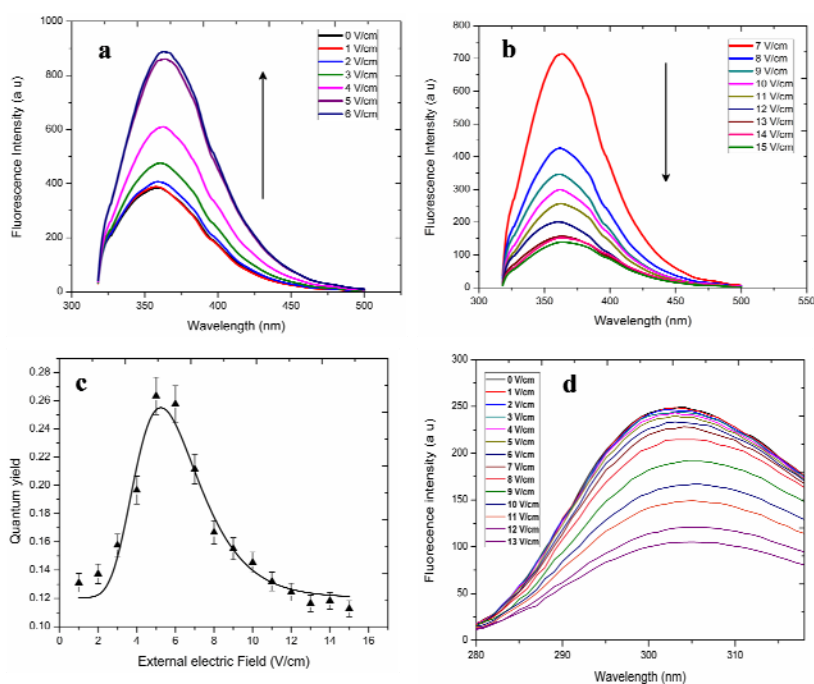


Fig. 2 The effect of EEF on photo-excitation of i) tryptophan: **a)** enhancement in fluorescence intensity at application of EEF from 0.0 to 6.0 V/cm; **b)** changes of fluorescence intensity at high strength from 7.0 to 15.0 V/cm; and **c)** change in the quantum yield a function of applied EEF, and non-linear curve fitting was applied to the graph; and ii) tyrosine **d)** reduction in fluorescence intensity with increasing electric field amplitude.

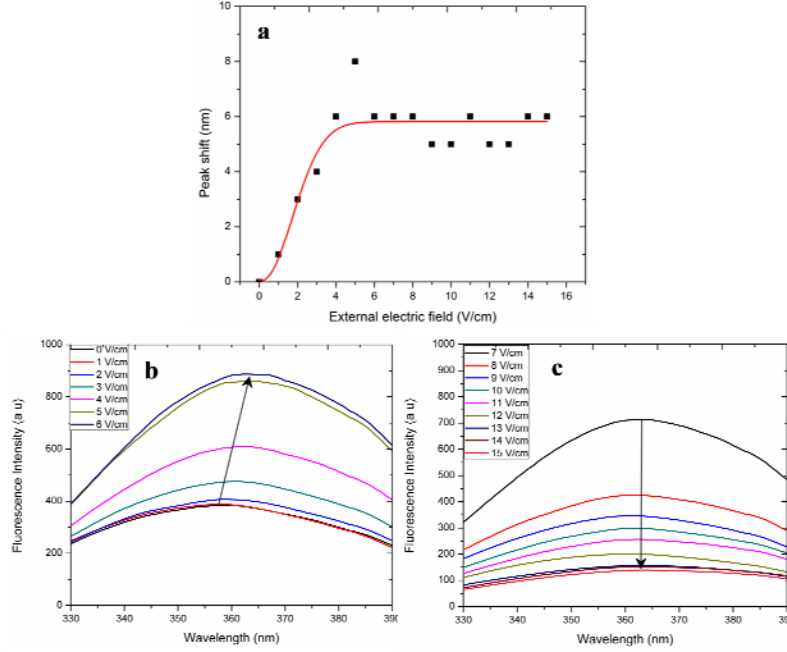


Fig. 3 a) Illustration of the influence of EEF application to the emission peak shift of the tryptophan amino acid solution (the non-linear curve fitting was applied to the graph), b) spectral shift of tryptophan at application of EEF from 0.0 to 6.0 V/cm and c) no spectral change at higher EEF.

However, in the present case, there is an absence of quencher that usually forms a complex/bind; hence, it could be possible to infer that the decrease in fluorescence occurs due to interference of higher applied field. Therefore, the EEF induced photo-excitation process may affect the molecular orientation, surface charge density, natural fluorescence decay rates, which may strongly influence the lifetime of the fluorophore. The intensity changes that are associated with fluorophore can be studied by the following equation that shows the direct relation between emission intensity and lifetime:

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} \quad (2)$$

where F_0 and F are the intensity of fluorophore in the absence and presence of external field and τ_0 and τ are the fluorescence lifetime of the fluorophore in the absence and presence of external field, respectively. The dynamic quenching is a rate process that depopulates the excited state as a function of field strength [9]. The decrease in the intensity takes place due to the process of depopulating excited states without fluorescence emission. From the above findings, we strongly believe

and state that the interference of EEF in photo-excitation may result in a change in the non-radiative decay process of fluorescent amino acids.

CONCLUSIONS

The present investigation shows the effect of EEF on real-time photo-excitation of Trp and Tyr using fluorescence spectroscopy. Trp molecules showed a significant change in fluorescence intensity with an increase in EEF. As a result of real-time photo-excitation under an electric field, Trp experienced an induced structural conformation (i.e., changes in molecular energy, bond length, charge distribution, and specifically dipole moments of biomolecules). Such conformation showed an increase in quantum yield of Trp as well as fluorescence reduction at higher voltages. Tyr strongly quenched on application of EEF, since they do not possess/induced change in dipole moment. Thus, we conclude that there could be changes in the fluorophore's lifetime under photo-excitation process. These induced modifications could be an interesting sensing application in the structure and cell biology.

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Competing interest: The authors declare that they have no competing interests.

The first two authors have equal contributions to this study.

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