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Short communication

## Unexpected light emission from tyrosyl radicals as a probe for tyrosine oxidation

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## A B S T R A C T

Tyrosine residues (Tyr) on proteins are a favoured site of one-electron oxidation due to their low one-electron reduction potentials. In this work, light-induced oxidation of Tyr residues was investigated using direct ionisation (via 266 nm light excitation) and sensitized photo-oxidation (by 3-carboxybenzophenone as sensitizer and 355 nm). Light emission (fluorescence) was observed at 410–440 nm as a result of Tyr oxidation. This novel light emission process is shown to be dependent on the solvent and aromatic ring substituents, however it does not depend on pH. It is proposed, that after initial formation of tyrosine phenoxyl radicals (TyrO<sup>•</sup>) by one electron-oxidation, the TyrO<sup>•</sup> absorbs a second photon to give an excited state species that undergoes subsequent light emission. The intensity of this emission depends on the Tyr concentration, and the detection of this emission can be used to identify and quantify one-electron formation of oxidized Tyr residues on proteins.

## 1. Introduction

Oxidation of amino acid side chains in proteins is important in both normal human metabolism, and also in a wide range of diseases [1]. Redox reactions of specific amino acids are critical to signalling within and between cells, and a particular focus has been placed on the modification of Cys residues in phosphatases, kinases, peroxiredoxins, GSH peroxidases and transcription factors [2]. However, modifications at other residues, including tyrosine (Tyr) residues are both widespread and important, with both phosphorylation and sulfation occurring at the –OH groups of the phenol side chain.

As a consequence of the low one-electron reduction potential of the substituted phenol ring, Tyr residues are favoured sites of one-electron oxidation and can act as ‘radical sinks’ within proteins, with this resulting in the formation of (long-lived) Tyr phenoxyl radicals, TyrO<sup>•</sup> [3]. Oxidation of Tyr residues is of potential importance in modulating the activity of tyrosine-dependent kinases and phosphatases, as alteration to these residues modulates the formation of phosphorylated (and to a lesser extent sulfated) Tyr residues [4]. TyrO<sup>•</sup> are also key intermediates in photosystems (e.g. photosystem II) [5], in the catalytic activity of cellular enzymes (e.g. the synthesis of 2-deoxynucleosides by

ribonucleotide reductases) [6], and in the formation of structural biological polymers via radical-radical dimerization reactions to give di-tyrosine and higher polymers. Thus, Tyr oxidation is critical to the formation of insect cuticles [7], biological adhesives [8], the fertilization envelopes of sea urchins and oocyst walls of parasites [9].

The formation of di-tyrosine from dimerization of two TyrO<sup>•</sup>, a process that occurs with a rate constant close to the diffusion limit, has also been implicated in multiple human pathologies (e.g. Alzheimer's [10] and Parkinson's [11] diseases, and the formation of age pigments such as lipofuscin [12]). It has also been utilized in surgical wound closure procedures (photochemical tissue bonding, PTB) using light-induced protein cross-linking, in the presence of a sensitizer, as a protein ‘glue’ [13]. These data indicate both the importance of Tyr oxidation and the need to understand the photochemistry of Tyr and TyrO<sup>•</sup>. In the study reported here, we describe a novel laser-induced light emission (two-photon phenomenon) of transient species derived from the Tyr side chain photo-oxidation in model compounds, amino acids/peptides and proteins. This emission of light may be a rapid and convenient method of assessing formation of TyrO<sup>•</sup> in biological samples.

**Abbreviations:** AcN, acetonitrile; AcTyr, N-acetyltyrosine; 3CB, 3-carboxybenzophenone; Cys, cysteine; Gly-Tyr, glycytyrosine; HOCl, the physiological mixture of hypochlorous acid and its conjugate anion <sup>-</sup>OCl; HPPrAc, 3-(4-hydroxyphenyl)propionic acid; 3ITyr, 3-iodo-tyrosine; Lyso, lysozyme; MeAn, *para*-methylanisole; MeOH, methanol; pCr, *para*-cresol; Tyr, tyrosine; TyrA, tyramine; TyrO<sup>•</sup>, tyrosyl phenoxyl radical

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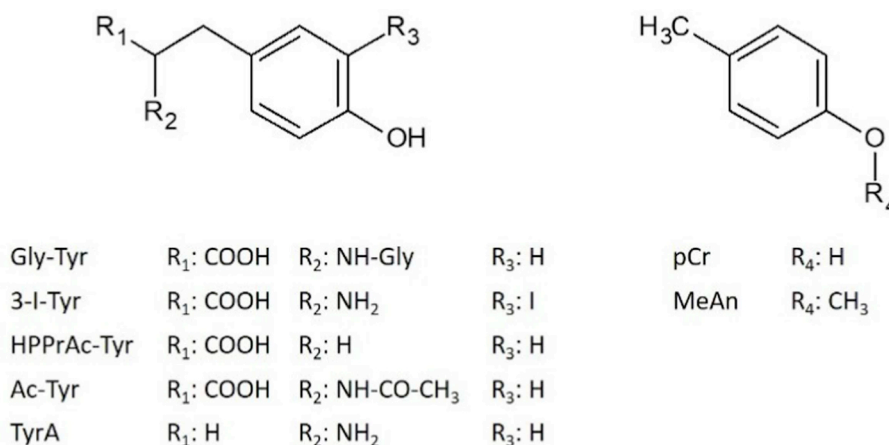


Fig. 1. Structures of the compounds containing the Tyr side chain and substituent aromatic rings, investigated in this study.

## 2. Materials and methods

### 2.1. Reagents

All chemicals were of high purity and obtained from Sigma Merck. High purity water was obtained from a Milli-Q system (Millipore, Bedford, MA). Samples were made up in phosphate buffer, pH 7.4 unless otherwise indicated (methanol, ethanol, acetonitrile).

### 2.2. Photo-oxidation of amino acids and related compounds in the presence of 3-carboxybenzophenone (3CB)

Light emission from TyrO<sup>•</sup> was observed and investigated by nanosecond laser flash photolysis (LFP) [14] using a Nd:YAG laser to provide excitation pulses at 355 nm to samples containing 3CB (4 mM) in 100 mM PBS, pH 7.4, in the absence of O<sub>2</sub>. Experiments were also run in other solvents, and mixed solvent systems, including water: acetonitrile (AcN) systems with ratios from 1:0 to 1:1, and water: alcohol mixtures. The kinetic traces were recorded at 5 or 10 nm intervals to give absorption and fluorescence spectra at various defined time points after the laser flash, with the data used to generate time-resolved spectra.

Confirmation that experiments as to the source of the light emission was obtained using the experimental system illustrated in Supplementary Fig. 1, in which the samples were excited by a continuous wave (CW) laser with a 355 nm excitation beam, coupled to a blue-ray diode laser providing extra pulses at 405 nm. In this system the 355 nm light beam selectively excites the sensitizer molecule (3CB), while the 405 nm laser excites the transients formed in the primary photo-oxidation reactions.

### 2.3. Photo-oxidation amino acids and related compounds by direct UV exposure

Light emission from TyrO<sup>•</sup> was observed and investigated by nanosecond laser flash photolysis (LFP) [14] using a Nd:YAG laser to provide excitation pulses at 266 nm to samples in 10 mM PBS, pH 7.4, in the absence of O<sub>2</sub>, with no added sensitizer. The absorption and fluorescence spectra were recorded at various defined time points after the laser flash, with the data used to generate time-resolved spectra.

### 2.4. Oxidation of lysozyme with HOCl

Lysozyme (Lyso, 2 mg mL<sup>-1</sup>, 140 μM) was treated for 30 min with 0–6 mM HOCl in phosphate buffer (10 mM, pH 7.4). The reaction was stopped by the addition of excess *N*-Ac-Cys (10 mM) which rapidly

reacts with HOCl, before recording of the fluorescence spectra.

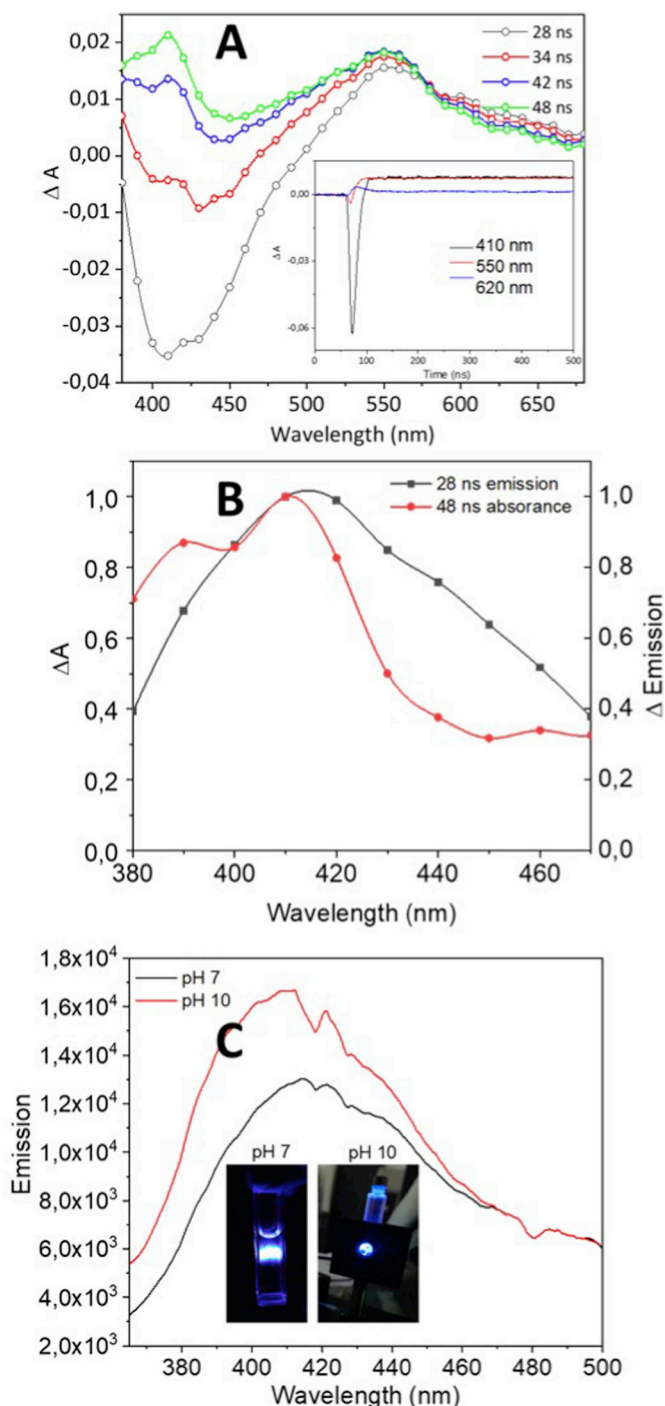
## 3. Results

Light emission from TyrO<sup>•</sup> was observed and investigated by nanosecond laser flash photolysis (LFP) [14] using a Nd:YAG laser with both absorption and fluorescence spectra recorded. A second experimental system consisting of two lasers and a spectrophotometer was constructed and employed to confirm that the light emission comes from transients derived from excited TyrO<sup>•</sup> (Supplementary Fig. 1).

Seven commercially-available compounds containing phenolic groups were investigated (Fig. 1), together with lysozyme as a model protein. Samples were diluted in different solvents and solvents mixture (phosphate buffer, PBS; methanol, MeOH; acetonitrile, ACN; water; water:ACN, and ACN:cyclohexane). 3-carboxybenzophenone (3CB) was used as a photosensitizer to generate TyrO<sup>•</sup> from Tyr [15]. During 3CB-sensitized laser flash photolysis of Gly-Tyr (λ<sub>ex</sub> 355 nm, 100 mM PBS, pH 7.4) a transient decrease in optical density (ΔA) was observed at short time periods (~10 ns) after the laser pulse over the wavelength range 380–490 nm (Fig. 2a).

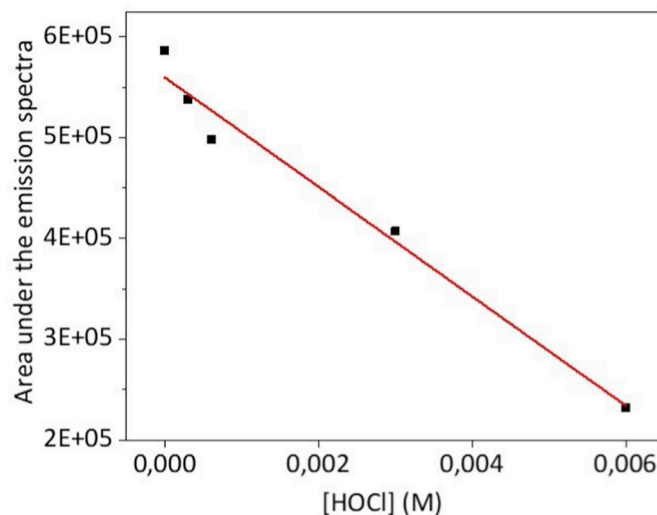
The analysis of kinetic traces recorded every 5 or 10 nm indicate the presence of an emission spectra with a maximum at 410 nm and shoulder at 430 nm, that has a very short life time (shorter or comparable to the laser pulse, ≤ 10 ns). This emission band decayed yielding an absorption band with a maximum at 410 nm, assigned to the Tyr phenoxyl radical (TyrO<sup>•</sup>) on the basis of literature data [15]. A similar emission band was observed over a wide range of Gly-Tyr concentrations (1–20 mM) with an increased intensity of emitted light. The broad absorption band at 550 nm is assigned to the 3CBH<sup>•</sup> ketyl radical on the basis of literature data [14,16]. Normalized data from time resolved emission (solid black line with squares) and absorption spectra (red line with circles) recorded over the time range with negative value of ΔA are presented in Fig. 2b. The emission spectrum is shifted from the TyrO<sup>•</sup> absorption spectrum. The purity of the parent Gly-Tyr was examined by HPLC-MS, UV-Vis and fluorescence spectroscopy, with none of these methods showing the presence of di-tyrosine or any other materials with known fluorescent properties in the investigated region. The effect of higher pH values on light emission from TyrO<sup>•</sup> is presented in Fig. 2c. The emission spectrum was not influenced by higher pH values, possibly because TyrO<sup>•</sup> can be readily formed over this pH range either from the neutral or ionized phenol.

The role of the phenolic ring in generating the observed fluorescence was examined using *N*-acetyltyrosine (Ac-Tyr) and 3CB in deoxygenated PBS (pH 7.4). A similar negative signal was observed in time-resolved traces between 380 and 490 nm. A range of related compounds (Fig. 1) were employed to examine the potential effects of



**Fig. 2.** (a) Time resolved spectra from kinetic traces for the sample of Gly-Tyr and 3CB, 28, 34, 42 and 48 ns after the laser pulse. Inset: kinetic traces recorded for 3CB (4 mM) and dipeptide Gly-Tyr (8 mM) in PBS (1 mM, pH 7.4) at 410 nm, 550 nm and 620 nm in the absence of  $O_2$ . (b) Time resolved spectra from kinetic traces for Gly-Tyr (8 mM) photo-oxidized by UV light in the presence of 3CB, after 28 ns (black line and symbols) and 48 ns (red line and symbols) after the laser pulse, with the data normalized. (c) Emission spectra (recorded using the experimental system outlined in [Supplementary Fig. S1](#)) for 5 mM Gly-Tyr with 4 mM 3CB (Ar-saturated, aqueous solution) at pH ~7 and 10. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the amine and carboxylate groups on the observed fluorescence. In each a negative signal  $\Delta A$  was detected in the laser flash experiments. The absence of either the amine group (3-(4-hydroxyphenyl)propionic



**Fig. 3.** Dependence of the fluorescence intensity detected at 430 nm on the extent of Lyso oxidation ( $2 \text{ mg mL}^{-1}$ ,  $140 \mu\text{M}$ ) induced by HOCl (0–6 mM). Samples were prepared in phosphate buffer, pH 7.4, with fluorescence spectra recorded both before the addition of HOCl and also 30 min after, with the reaction stopped by the addition of excess N-Ac-Cys to remove unreacted HOCl.

acid), or the carboxylic group (tyramine) did not influence the light emission by the transient derived from the phenol moiety. Para-cresol (pCr) also showed light emission ([Supplementary Fig. S2](#)).

In contrast, a number of other compounds, (e.g. methionine, histidine and tryptophan derivatives) lacking the phenol moiety did not emit light under the same conditions. 4-methylanisole (MeAn) ([Supplementary Fig. S2](#)) did not yield light emission over a wide concentration range. Thus, the light emission appears to be connected with the presence of free  $-OH$  group and does not appear to be technical artefact. However, three absorption bands were visible in the deconvoluted spectra for the MeAn system at 445 nm, 520 and 560 nm. The bands at 520 and 560 nm are assigned to 3CB transients, while the band at 420 nm is assigned to the MeAn radical anion.

The dipeptide Gly-Tyr was also irradiated with a CW laser in the presence of 3CB ([Supplementary Fig. S1](#)) to obtain TyrO $\bullet$ . This radical was then excited with a diode laser at 405 nm (where it has a significant absorbance). This resulted in an increase in emitted light intensity but no change in the spectra shape. This observation suggests that the emitted light comes from TyrO $\bullet$ .

The role of the solvent in the observed photochemistry was also examined. Non-protic LC-MS purity solvents did not give rise to the light emission detected with protic solvents, indicating a key role for solvent protons in the emission process. Laser flash experiments were also carried out using mixtures of Gly-Tyr and 3CB in different water:AcN ratios from 1:0 to 1:1. A change in the emission maximum from 405 to 430 nm, and the shape of the emission spectra, were observed with increasing amounts of AcN compared to water. The effect of the solvent on the observed emission was also examined for all the other compounds. Spectra were also recorded in MeOH and EtOH and mixtures of these with water, with light emission detected for all of the alcohol: water ratios examined. These observations indicate that solvent protons also play a crucial role in the formation of the light emitting transient, with the generation of TyrO $\bullet$  in non-protic solvents that was insufficient to induce light emission.

To determine whether this light emission can be employed to probe the oxidation of surface exposed Tyr residues in proteins, studies were carried out with the model protein lysozyme (Lyso) using a similar laser/3CB system. The time resolved spectra obtained from deoxygenated aqueous solutions of Lyso solution with 3CB at 355 nm also contained clear negative signals from TyrO $\bullet$  ([Supplementary Fig. S3](#)).

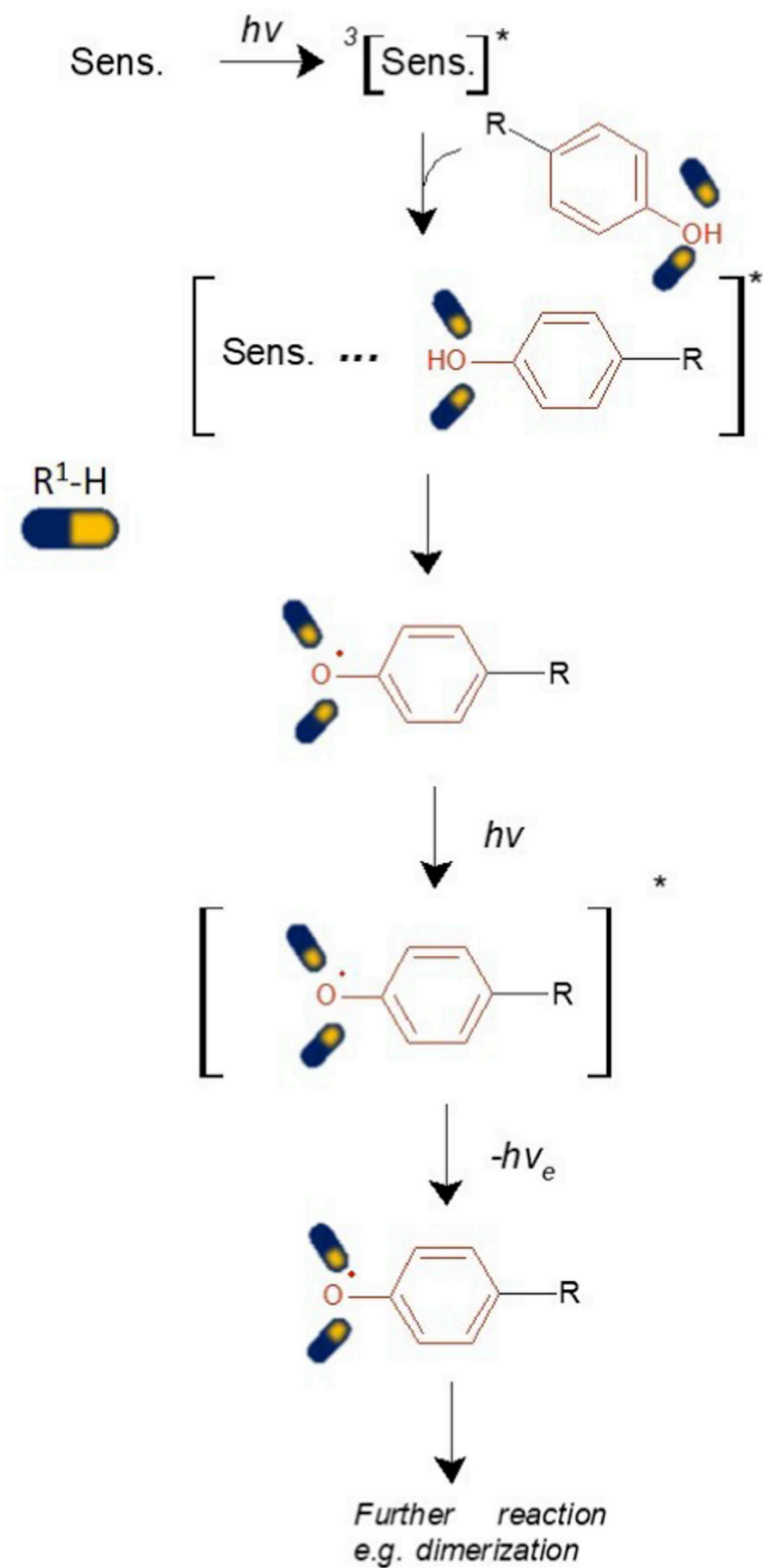


Fig. 4. Proposed mechanism for the formation of TyrO $\cdot$  via laser flash photolysis (LFP) with 3CB as sensitizer, complex formation with protic solvent molecules, and subsequent reaction. R – different substituents for Tyr model compounds and protein chain. R1 – protic solvent e.g. water, MeOH.

Interestingly, long term photooxidation of Lyso at 355 nm resulted in the disappearance of the fluorescence signal due to depletion of the parent Tyr and the formation of the product species di-tyrosine. A similar effect was seen when Lyso was pre-treated (oxidized) with different concentrations of HOCl, with the fluorescence of TyrO<sup>•</sup> decreased with increasing concentrations of prior oxidation (Fig. 3). The corresponding phenoxy radical formed from 3-chlorotyrosine generated by HOCl does not emit light, an observation confirmed by direct laser flash photolysis of authentic 3-chlorotyrosine. Similar behaviour was detected with 3-iodotyrosine. In this case a heavy atom effect may be responsible for fluorescence quenching.

Laser flash photolysis was also carried out for all of the investigated compounds without 3CB, using direct excitation at 266 nm. The negative signals in the kinetic traces and the emission spectra were similar to the excitation at 355 nm with 3CB as photosensitizer. These data suggest that light emission from TyrO<sup>•</sup> occurs irrespective of the mechanism of formation of this species [3].

#### 4. Discussion

The light emission (fluorescence) phenomena observed in these studies can be rationalized by the processes presented in Fig. 4. The 3CB molecule absorbs light (355 nm) yielding the excited triplet state 3CB\* that is quenched by the Tyr side chain. As a result of electron transfer from Tyr to 3CB, TyrO<sup>•</sup> is formed. It is proposed that this radical forms a complex with protic solvent molecules, and that the observed light emission arises from the decay of this complexed excited TyrO<sup>•</sup> species. It should be noted that the observed fluorescence occurs via a two photon-phenomenon, i.e. that within the same laser pulse, the sample is excited, forms TyrO<sup>•</sup>, and absorbs a second photon to excite the radical. Theoretical calculations indicate the presence of a low energy electronic state with symmetry A<sup>+</sup> that is shifted to a higher value in the presence of two water molecules. Further theoretical calculations would appear to be warranted on this system [17].

The detection of this rapid light emission from TyrO<sup>•</sup> may be a convenient and rapid method of examining the formation and fate of these key intermediate radicals on proteins in biological systems. The lifetime and wavelength maximum of this emission process may also provide a new tool for examining the environment and reactions of TyrO<sup>•</sup>, in a similar manner to the use of Trp fluorescence as a probe for the environment of this residue. Additional studies are required to address the full implications and possibilities of this novel photo process.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.freeradbiomed.2020.03.022>.

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