

Single Molecule SERS Leads to the Observation of the Intermediate Form of Green Fluorescent Protein Chromophore

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ABSTRACT

The protonated (P) form of the green fluorescent protein (GFP) involves a protolytic reaction under violet-blue light by forming an excited anion of the intermediate form of the chromophore from the photo-excited protonated form. This study reports the observation of this proton movement cycle by acquiring vibrational spectra of single GFP molecules using “nanometal-on-semiconductor” SERS substrates and applying a “pump and probe” technique. A new peak at 1510 cm^{-1} prominently exists with 1560 cm^{-1} (P) peak. Statistical analysis of the GFP population reveals a higher probability (about 9 times) of observing both 1510 cm^{-1} and 1560 cm^{-1} peak over the probability of finding both 1510 cm^{-1} and 1530 cm^{-1} (deprotonated) in one ‘spectral jump’. We recognize this unique Raman peak at 1510 cm^{-1} as a marker for the intermediate form of the chromophore. This peak is also observed both in ‘Cis’ (1260 cm^{-1}) and ‘Trans’ (1280 cm^{-1}) configuration of the chromophore, but considerably more with ‘Cis’ configuration.

Keywords: Single molecule, SERS, GFP, Raman, Förster cycle.

1 INTRODUCTION

Wild type green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* has achieved a widespread fame for being an exceptionally exploitable biological marker in the living systems due to its formation of a stable chromophore without requiring any external cofactors [1]. The complex absorption spectra of GFP exhibits two major peaks; one at 395 nm , associated with the protonated form (A) of the chromophore and another at 475 nm , associated with the deprotonated form (B) [1]. Exciting either of the protonated or deprotonated forms, that is present approximately in a 6:1 ratio, results in highly efficient green fluorescence (508 nm) [1]. This kind of excited state protolytic reaction which forms excited anion from a protonated species is well established in solution photochemistry and was discovered by Theodor Förster [2].

Excited state dynamics of the GFP chromophore studied by Chatteraj et al. [3] concluded that excited state proton transfer (ESPT) is involved in photoconversion from A to B via an excited intermediate state I^* (Fig. 1(a)). This study [3] strongly supports the existence of Förster cycle in GFP (Fig. 1(b)). Exciting the ground state of the A form creates a photo-excited protonated form (A^*) that is converted to an intermediate excited anion (I^*) on a picosecond (ps) time

scale [3-7]. I^* quickly relaxes back to the intermediate ground state (I) giving green emission, which eventually repopulates the protonated species of the GFP chromophore [3]. This photo-excited conversion mechanism ($A \rightarrow A^* \rightarrow I^* \rightarrow I \rightarrow A$, Förster Cycle, Fig. 1(b)) is caused by ESPT (proton movement) from the chromophore to the nearby amino acid residues [3, 4]. Conversion of $I^* \rightarrow B^*$ happens very rarely and exciting the B form directly creates B^* (excited anion) which relaxes back to the ground state by a similar green emission as in $I^* \rightarrow I$ [1, 3].

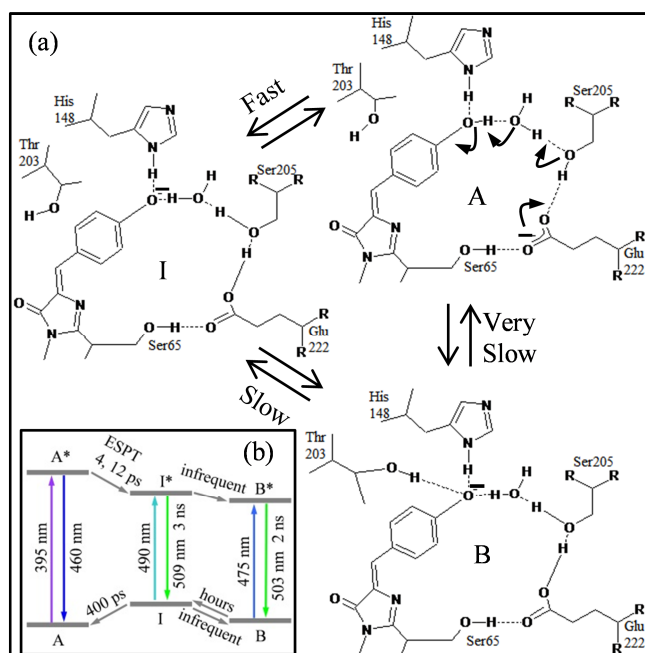


Figure 1: (a) Suggested photoconversion mechanism of the A and B forms of the GFP chromophore through I state. The conversion from protonated (A) form to the anionic (B) species involves phenolic proton movement of Tyr66 via an extended hydrogen bonding to the carboxylate oxygen of Glu222. Arrows indicate the ‘proton pathway’ in opposite direction [8, 9]. (b) Photodynamics of wild type GFP indicating Förster cycle. Relaxation times for the excited states are also included [4-7].

Conversion of $B \rightarrow I \rightarrow A$ is also very rare [3], which suggests that the certain protolytic reaction discovered by Förster is mostly characteristic to the protonated form (A) of the GFP chromophore. Creemers et al. [10] later distinctly identified the A, B and I states using hole burning spectroscopy. X-ray crystallographic analysis indicates that the chromophore in wild type GFP is present in cis-

configuration [11, 12], that is protected centrally by an 11-stranded β barrel forming a cylinder of 4.2 nm long and 2.4 nm in diameter [12]. This protective ‘jar’ like structure of proteins is crucial for the fluorescence, thus it has been suggested that cis configuration of the chromophore corresponds to the ‘bright’ state while trans being the supposed ‘dark’ state [11, 13]. Cis-trans photoisomerization has been verified experimentally for HBDI, a synthetic analogue of the bare wild type GFP chromophore [13]. It has been proposed that along with the dominant proton movement step associated with the main structural change for GFPs, a volume conserving cis-trans isomerization mechanism can also be involved as a part of this complex photodynamic behavior of the chromophore embedded in the protein jar [5, 11, 13].

Utilization of Surface-Enhanced Raman Scattering (SERS) to detect single molecules (SM) [14, 15] opened up an exciting opportunity to monitor detailed molecular dynamics in heterogeneous media, from living cells to chemical catalysts including protein structures [16]. Habuchi et al. [17] exploited SM SERS to observe the dynamic behavior of EGFP between a protonation and deprotonation state. Later Singhal and Kalkan [18] reported a uniquely successful demonstration of SM SERS to show conformational changes in yellow fluorescent protein.

In the current study, by using SM-SERS, we observe the Förster cycle at SM level by exciting the dominant absorption band of GFP at 395 nm. Intense optical pumping due to the enhanced field of nanoparticles facilitates the observation of the intermediate state (I) at the resolution of individual molecules. The ‘I’ state is observed as the overall inhabiting time of the GFP chromophore in its native protonated form is lowered and relative inhabiting time of ‘I’ form in the Förster cycle is increased. Furthermore the intermediate state of the chromophore is identified both in cis and trans configurations depending upon the Raman markers rationally inferred from the study of two red fluorescent proteins, namely DsRed and eqFP611 respectively [19]. Depending on the well-established protonation cycle of GFP and a strongly suggested association of cis-trans photoisomerization of the chromophore leads to the recognition of ‘I’ state in a prominent cis configuration. Realizing the information that both cis configuration and excited ‘I^{*}’ state are associated with the fluorescence of the GFP chromophore further supports that Förster cycle is being driven by the external pumping source.

2 MATERIALS AND METHODS

In this study we prepared our ‘nanometal on semiconductor’ SERS substrates by reducing Ag^+ on Ge thin films [20]. 1 μL aliquot of wild type GFP solution (10^{-9} M, pH 7) is spotted on the SERS substrate and inserted into an optical cell (Starna cell; $12.5 \times 3.5 \times 45$ mm³). Subsequently, the cell is sealed with a small piece of wet wipe. Thus the aliquot does not dry quickly and the same

SERS substrate with the same aqueous GFP aliquot is used for a relatively long time.

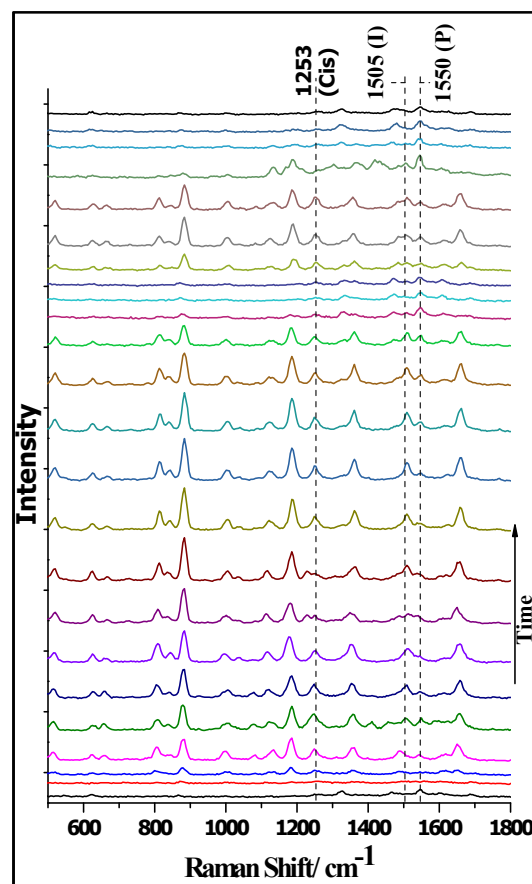


Figure 2: Time series SERS spectra showing a “spectral jump”. Initially a weak background is observed. But when a single GFP molecule occupies a highly enhanced SERS site then strong and well resolved peaks appear. These peaks are sustained for several spectra before the molecule diffuses out of the “hotspot”. These spectra (100 ms interval) indicate both intermediate (I) and protonated (A) state of the GFP chromophore in cis configuration (100 μW laser power).

Next, a 532 nm Nd:YAG is used as the Raman probe and simultaneously pumping is achieved by a 405 nm external LED source. Once a Raman measurement is started, single molecule spectra are monitored as a repeated observation of appearance and disappearance of strong and clearly resolvable peaks over a weak background (Fig. 2) [18, 21]. Raman acquisitions are carried out using WITec alpha300R system, and Prizmatix fiber coupled triple LED light source is used for the pumping. These temporal “spectral jumps” occurring in every several seconds are associated with single GFP molecules diffusing in and out of a highly enhanced SERS site [14, 15] (i.e. a “hotspot”) with an average inhabiting time of 1 s or less as depicted by Fig. 2. SERS is acquired as continuous time series spectra at 100 ms intervals under 100 μW laser power.

3 RESULTS AND DISCUSSION

Our recognition of the captured SERS spectra as being collected from single GFP molecules can be supported by the following observations, (a) absence of statistical averaging and appearance of sharply resolvable peaks, (b) sudden appearance and disappearance of Raman peaks in a continuous spectral acquisition, (c) minor temporal fluctuation of spectral wavenumbers for the Raman peaks, (d) relative intensity fluctuation of the peaks and (e) sudden appearance of a certain peak with a dramatic loss of intensity or complete disappearance of some other peaks (structural transition) [15, 22-25]. Also, use of very low concentration (10^{-9} M) of aqueous GFP solution confirms that, these spectra capture the photodynamics from single molecules arrested in one ‘spectral jump’.

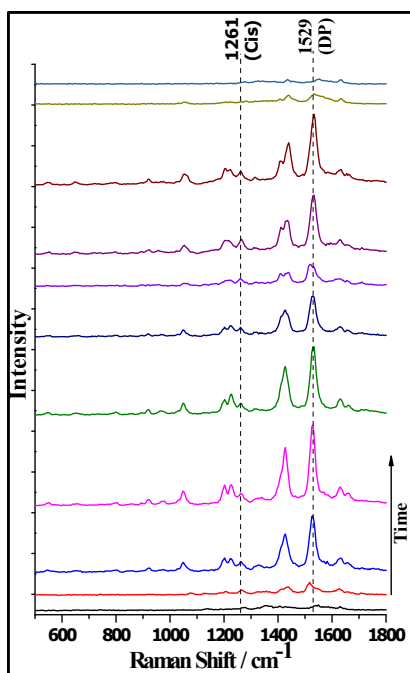


Figure 3: Time series SERS spectra indicating the absence of “I” state in the presence of deprotonated (B) state of the GFP chromophore in Cis configuration (100 ms integration time and 100 μ W laser power).

Fig. 2 displays such time series spectra captured from single GFP molecules, while they temporarily enter and occupy ‘high SERS enhancement’ sites. These spectra clearly demonstrate the dynamic behavior of the GFP chromophore under photo excitation. Protonated Raman marker at 1550 cm^{-1} [17] and Raman peak for cis configured GFP chromophore at 1260 cm^{-1} [19] are clearly observable in these spectra. Apart from these two established markers, a new Raman peak at 1505 cm^{-1} ($\pm 5\text{ cm}^{-1}$) is also identified. Thus the captured GFP molecule is present in cis and protonated form, which corresponds to state ‘A’ according to Fig. 4(a). This newly identified peak is found to be consistently absent without the external 405 nm LED pumping source (data not shown).

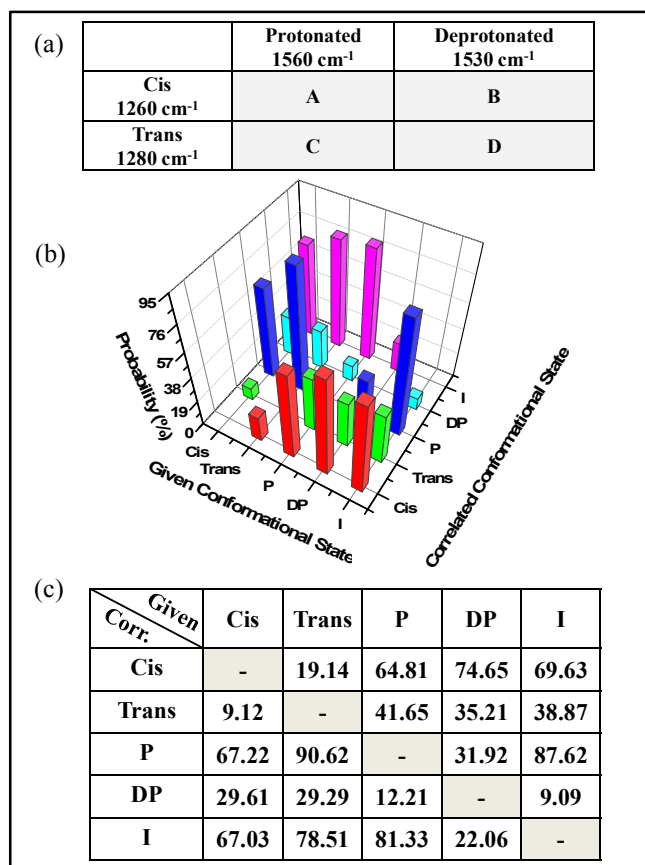


Figure 4: (a) Adopted vibrational (Raman) markers in the present study for characterizing the main four conformational states of GFP chromophore. (b) Probability of capturing different conformational (Cis, Trans, Protonated, Deprotonated, Intermediate) states of GFP chromophore for a given conformational state. (c) Statistical analysis of the GFP population of 64 ‘spectral jumps’ consisting a total of 712 spectra.

In the SM ‘spectral jump’ of Fig. 3, Raman marker of deprotonated GFP chromophore is observed at 1529 cm^{-1} [17]. These spectra also exhibit Raman peak for cis configured chromophore at 1261 cm^{-1} [19] confirming the chromophore being in the state ‘B’ adopted in Fig 4(a). Remarkably, aforementioned new Raman peak at around 1505 cm^{-1} is clearly absent in these series of spectra. This observation is found to be very consistent throughout our experiments.

These pivotal observations can yield an explanation for the photophysical behavior of single GFP molecules monitored in our study by considering, (a) selection of an external LED as a pumping source with a very close wavelength (405 nm) to the dominant absorption band (395 nm) [1] enables excitation of state ‘A’, i.e. mainly the protonated form of the chromophore; (b) achievement of intense optical pumping due to the surface enhancement by the nanoparticles [26-28], considerably lowers the overall inhabitation of the chromophore in its protonated form.

Thus using an external LED source (405 nm) to drive the continuous excitation of the 'A' form of the chromophore and utilization of the favorable conditions induced by highly enhanced SERS sites; pumps state 'A' very quickly to reduce its overall inhabiting time. This in return increases relative inhabitation of the chromophore in 'I' state.

The Raman peak at around 1510 cm^{-1} is prominently existent with the protonated (1560 cm^{-1}) form of the chromophore and significantly absent with the deprotonated (1530 cm^{-1}) form. Statistical analysis of the GFP population, included in Fig. 4(b), reveals an interestingly higher probability (approximately 9 times, Fig. 4(c)) of observing 1510 and 1560 cm^{-1} peak together over the probability of finding both 1510 cm^{-1} and 1530 cm^{-1} in one 'spectral jump'. This Raman peak at around 1510 cm^{-1} is found to appear both with the Cis (1260 cm^{-1}) and Trans (1280 cm^{-1}) Raman markers of GFP but noticeably more with the 1260 cm^{-1} . Dominant presence of the peak at 1560 cm^{-1} and considerable absence of 1530 cm^{-1} peak while considering 1510 cm^{-1} leads to the realization and identification of this peak as a unique vibrational Raman marker for the intermediate form (I) of the GFP chromophore, which has the same conformation as 'A' form and same protonation state as the 'B' form but differs by a conformational change mostly at Thr203.

4 CONCLUSIONS

Based upon our extensive SM SERS acquisitions a unique vibrational mode at around 1510 cm^{-1} is identified as a new Raman marker for the intermediate state 'I' of the GFP chromophore. These series of evidences are the first reported document of observing the 'I' state using vibrational spectroscopy and also the resolution is achieved at a single molecule level. Observation of this 'I' state becomes possible as the overall inhabiting time of the chromophore in its protonated form (A) is significantly reduced, which in turn facilitates a relative increase in the inhabiting time of the intermediate state (I).

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