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Drop Coating Deposition Raman Spectroscopy of Fluorescein Isothiocyanate Labeled Protein

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Abstract

Using bovine serum albumin (BSA) as the model protein normal Raman spectra of Fluorescein isothiocyanate (FITC) -conjugated protein was systematically studied for the first time using both solution and the drop coating deposition Raman (DCDR) sampling techniques. The FITC-BSA Raman spectra are dominated by the FITC Raman features that are strongly pH dependent. Current DCDR detection sensitivity obtained with a 10:1 FITC-BSA conjugate is 45 fmol in terms of total protein consumption and ~15 attomol at laser probed volume. Unlike the FITC-BSA solution Raman spectra where the FITC Raman features are photostable, concurrent FITC fluorescence and Raman photobleaching is observed in the DCDR spectra of FITC-BSA. While the FITC Raman photobleaching follows a single exponential decay function with a time constant independent of the FITC labeling ratio, the fluorescence background photobleaching is much more complicated and it depends strongly on the FITC labeling ratio and sample conditions. Mechanistically, the FITC Raman photobleaching is believed to be due to photochemical reaction of the FITC molecules in the electronically excited state. The FITC fluorescence photobleaching involves both concentration quenching and photochemical quenching, and the latter may involve a photochemical intermediate that is fluorescence inactive but Raman active.

Keywords

Fluorescein isothiocyanate (FITC); Bovine serum albumin (BSA); DCDR; Photobleaching

INTRODUCTION

Protein and dye conjugation is probably the most widely used strategy in biomedical and biological research.^{1–4} Various fluorescence-based bioanalytical and biophysical techniques have been developed for studying protein folding, antibody-antigen binding and comparative proteomics quantification, etc.^{4–6} By developing fluorophores that react specifically with modified amino-acids, fluorophore tagging has been explored for quantification of protein posttranslational modifications including protein phosphorylation, glycosylation and oxidation.^{7,8}

With its extended π conjugation electrons, fluorophores usually have large Raman cross-sections and can potentially serve as an effective Raman tag for Raman spectroscopic

detection of proteins and protein modifications. Recently, we demonstrated a drop coating deposition Raman based (DCDR) ratiometric Raman spectroscopic technique for quantitative determination of protein oxidative damage.⁹ By conjugating dinitrophenyl hydrazine, a protein carbonyl reactive chromophore, with oxidized model proteins including bovine serum albumin and lysozyme, protein oxidative levels were quantified with sub pmol protein carbonyl.⁹ One possible strategy to further enhance the detection sensitivity of this ratiometric Raman technique is to explore the use of more Raman active reagents, such as fluorescein dyes, as Raman tags.

In this work, we wish to characterize the DCDR spectroscopic characteristics of fluorescein-labeled proteins using fluorescein isothiocyanate (FITC) conjugated bovine serum albumin (BSA) as examples. Because of its large fluorescence quantum yield and the excellent reactivity of its isothiocyanate group with the primary amine in protein, FITC has been used extensively for in-vivo and in-vitro biomolecule detections.^{10–12} Fluorescein based reagents have also been developed for fluorescence detection of protein modifications.^{13–15} For example, fluorescein-5-thiosemicarbazide is a commonly used reagent for glycoprotein and protein carbonyl detection.¹⁶

DCDR is a sensitive Raman detection technique with which high quality Raman spectra can be routinely obtained with label-free protein samples of low μM concentrations and/or pmol quantity.^{17–19} To date, DCDR has been applied for study of protein-ligand binding,^{18,20} protein fibrillation,²¹ protein post-translational modification,^{9,22,22} and quantitative resolution of protein mixtures.²³ Compared to fluorescence spectroscopy that usually has very broad spectral features thus with limited molecular specificity, Raman spectrum is much more informative, with the ability to differentiate molecules with high structural similarity. As a result, studying the Raman spectral characteristics of fluorophore labeled protein can be important in bioanalytical and biophysical applications where the molecular specificity is often desirable. While SERS study of fluorophore labeled protein has been attempted.^{24,25} To our knowledge normal Raman spectroscopy of fluorophore-protein conjugate has not been systematically investigated.

EXPERIMENTAL

Chemicals and equipment

FITC was purchased from Fluka. The FITC conjugated BSA with a labeling ratio of 10 was purchased from Sigma-Aldrich and will be referred to as 10FITC-BSA hereafter. BSA was also purchased from Sigma-Aldrich. RamChip slide used for DCDR spectral acquisition was obtained from Z&S Tech. The 10X concentrated phosphate buffer saline (PBS) was purchased from Fisher-Scientific and was appropriately diluted before use. A 0.1M carbonate-bicarbonate buffer at pH 9.7 was prepared for protein-FITC conjugation. Nanopure water was used in all the experiments. Dialysis membrane (MWCO=3500 Dalton) and ultrafiltration tubing (MWCO=5000 Dalton) was purchased from Spectrum and Millipore respectively.

UV-Visible measurements were carried out on a Thermo Fisher Scientific Evolution 300 UV-Visible spectrophotometer. Ultrafiltration of the FITC/protein conjugation solution was carried out using a Fisher Scientific 21000 R centrifuge. Raman spectra were acquired with a Horiba Jobin Yvon LabRam HR Raman microscope system equipped with a 632.8 nm HeNe excitation laser. Unless specified, all the solution Raman spectra were acquired with a 13 mW laser power before a 10X objective, while the DCDR spectra were obtained with 1.3 mW laser power before a 100 X objective that corresponds to a sampling spot of $\sim 2 \mu\text{m}$ in diameter. Raman shift was calibrated with a neon lamp and a wave number accuracy of $\pm 1 \text{ cm}^{-1}$.

Preparation of FITC-protein conjugates

Besides the 10FITC-BSA that was purchased from Sigma-Aldrich, FITC and BSA conjugates were also synthesized in house to investigate the possible dependence of Raman spectral characteristics on the FITC labeling ratios. FITC/BSA conjugations were performed based on published procedures.^{6,26,27} Briefly, equal volumes of 25 mg/ml of protein solution and 0.6 mg/ml of FITC solution, both prepared using a 0.1 M carbonate-bicarbonate buffer solution at pH ~9, were mixed and continuously shaken for ~4 hours. We ended the conjugation reaction by dialyzing the solution (Spectrapor, cut-off 3.5 kDa) against nanopure water followed with three ultrafiltrations. Membrane dialysis was carried out at 8 °C and the dialysis solvent (~450 mL) was changed three times for every 4 to 6 hours. Figure 1 shows the UV-Visible spectra obtained with the FITC/BSA conjugated solutions, where spectrum (a) is the purified in-house prepared FITC/protein conjugate and spectrum (b) is the commercial 10FITC-BSA. Spectrum (c) was acquired with the final filtrate solution of the in-house prepared FITC/BSA conjugate that contained molecules capable of passing through the ultrafiltration membrane. The absence of the FITC peak in the UV-vis spectrum of the filtrate indicates that the combination of membrane dialysis and ultrafiltration effectively removed all free FITC from the FITC conjugation solution.

The FITC labeling ratio was determined based on FITC vendors' instruction where the molar extinction coefficient of FITC at the 495 nm region was given as $84000 \text{ M}^{-1} \text{ cm}^{-1}$ and its absorbance at 280 nm was assumed to be 30% of the peak absorbance in the 495 nm region. Using protein molar absorptivity of $43291 \text{ M}^{-1} \text{ cm}^{-1}$ for BSA,²⁸ the FITC labeling ratio of the synthetic BSA/FITC conjugate was ~1. The synthetic BSA/FITC conjugate will be referred to as 1FITC-BSA from here on. For the sake of simplicity, FITC-BSA will be used as a general term for these two BSA/FITC conjugates. It should be noted that the UV-vis spectral profile of protein/FITC conjugates depends strongly on the FITC labeling ratio, which will inevitably introduce error into the estimation of the FITC labeling ratio. Such an error, however, will not affect the conclusion of our study.

pH dependence of DCDR and solution Raman Spectra

The pH dependence of FITC-BSA Raman spectral features was investigated with 10 FITC-BSA solutions. ~ 2 mg/ml of 10 FITC-BSA was prepared with 0.01 M PBS buffer (pH=7.5) and the solution was then adjusted to pH 4.9 and pH 9.1 using 0.1 N HCl or 0.1 N NaOH. Both solution Raman and DCDR spectra were acquired at each pH value. The signal integration time for the DCDR was about 50 s or 100 s with the laser power before the objective 1.3 mW while the solution Raman spectra is 600 s with a laser power of 13 mW.

Raman spectra of buffer solution was also obtained in order to subtract the buffer contribution to the solution Raman spectra of the 10FITC-BSA samples. It is important to note however, that no solvent or buffer background subtraction was needed for the DCDR spectra obtained with the FITC-BSA samples since all the DCDR spectra were obtained by focusing the laser beam onto the protein ring that was free of spectral interference from buffer molecules. Upon solvent evaporation, the FITC-BSA conjugates and buffer molecules were segregated onto different areas on the Raman substrate. This phenomenon has been called "sample segregation".²⁹ Another important note is that prior to its Raman spectral acquisition, the pH=4.9 10FITC-BSA solution was filtered with a 0.2 μm filtration membrane to remove the precipitated 10FITC-BSA since it has lower solubility at this pH. The lower solubility of 10FITC-BSA at pH=4.9 is not surprising since the isoelectrical point of BSA is 4.7,^{30,31} and it is commonly known that protein has its lowest solubility at its isoelectrical point. Also, fluorescein has much lower solubility in acidic solutions as it is mainly monoanionic at this pH. This in turn makes fluorescein less hydrophilic since it forms dianion at a higher pH.³²

DCDR detection sensitivity of FITC-BSA

A series of 10FITC-BSA solutions with concentrations of 15 μM , 1.5 μM and 0.15 μM respectively, were prepared using nanopure water, to investigate the DCDR detection sensitivity of FITC-BSA. Protein deposition was made with a Genomic Solutions ProPrep robotic station (Boston, MA) that was programmed to deposit sample volumes of 700 nl, 500 nl and 300 nl respectively onto a MALDI plate (ABI-01-192-6-AB, Applied Biosystems, Foster City, CA). Besides the 10 FITC-solutions, BSA solution (with no FITC conjugation) was also deposited onto the MALDI plate as a control. The protein deposits were dried in ambient conditions. Fluorescence imaging of the 10 FITC-BSA and BSA loaded plates was acquired with a 488 nm excitation wavelength and an emission wavelength of 500 nm.

Photobleaching of FITC-BSA

10 μM 1FITC-BSA and 10FITC-BSA solutions were prepared using nanopure water. For each sample, 10 μl of protein solution were deposited onto a RamChip substrate and let dry in an AirClean chamber (Raleigh, NC). DCDR spectral acquisition was conducted at ambient conditions. The effect of laser power on Raman photobleaching was investigated by adjusting the laser illumination power on a sample with a set of neutral density filters. It should be noted that because of difficulties in measuring the laser power after the 100X objective (NA=0.95), all the laser powers listed in this work are before objective, which is about 2 times higher than the laser power on the sample. With each experimental condition, a series of DCDR spectra were obtained sequentially on the same sampling spots under continuous laser illuminations. The photobleaching was monitored based on the Raman peak intensity of the 1186 cm^{-1} peak as a function of laser illumination time. Background intensity was obtained by averaging the baseline intensity at the left and right side of the 1186 cm^{-1} peak and was subsequently subtracted from the spectral intensity of the 1186 cm^{-1} Raman peak. It is important to note that the exact Raman peak and background intensity may vary depending on the chosen baseline pixels. However, such variations won't affect our time course of Raman intensity and spectral background change after sequential laser illuminations.

RESULTS AND DISCUSSION

Raman spectra of FITC-BSA

Figure 2 shows both solution and DCDR spectra obtained with 10FITC-BSA solutions at pH 9.1, pH 7.5 and pH 4.9 respectively. Evidently, the signal to noise ratio of the DCDR spectra of 10FITC-BSA is much better than the corresponding solution Raman spectra, despite the longer integration time for the latter. This is consistent with previous reports that DCDR is a much more sensitive Raman sampling technique.¹⁸ Another remarkable feature is the total dominance of the FITC Raman features. Indeed, in all of the 10FITC-BSA spectra, no protein Raman features including the most Raman active phenylalanine peak at 1003 cm^{-1} region, can be identified, even though there are 30 phenylalanine residues in each BSA molecule⁹ compared to the 10 FITC molecules conjugated to each protein. In fact, the FITC Raman features also dominate the DCDR of 1FITC-BSA. This result is not surprising given the extensive π conjugation electrons in the xanthene ring in the fluorescein structure. Also, similar to free fluorescein,^{32,33} the Raman spectral features of BSA-conjugated FITC exhibits a large pH dependence in both solution and DCDR spectra which is commonly attributed to the pH dependence of the FITC protolytic state. For example, the 1334 cm^{-1} peak in basic solution is shifted to 1313 cm^{-1} in the acidic condition, accompanying the blue shift of the benzoate peak from 1171 cm^{-1} to the 1186 cm^{-1} . Detailed peak assignment is shown in Table I which is obtained mainly based on the work by Wang *et al.* Comparison of our results with that by Wang *et al* reveals no significant differences in the peak positions (<

4 cm^{-1}) of the FITC Raman features before and after FITC conjugation. Consistent with previous results obtained with fluorescein at similar pH conditions, our solution Raman spectra of 10FITC-BSA indicates that at pH 4.9, most of the FITC are in a monoanionic form but are dominantly in a dianionic form at pH 9.1, while the shoulder peak in the 1313 cm^{-1} region in the pH 7.5 10FITC-BSA solution suggests a mixed fluorescein protolytic state.

While the solution and DCDR Raman spectra are very similar for both pH 4.9 and pH 7.5 10FITC-BSA solutions, they are markedly different for the pH 9.1 FITC-BSA sample, particularly in the spectral region of 1100 cm^{-1} to 1400 cm^{-1} . The small shoulder peak at the 1334 cm^{-1} region in the solution spectrum becomes much more evident in the DCDR Raman spectrum, indicating that some of the dianionic FITC in the 10FITC-BSA solution were converted into a monoanionic form in its DCDR deposits. The reason for this change is unclear. It is possible that the local pH and salinity of the FITC-BSA molecule in a DCDR deposit can be substantially different from its counterpart in a FITC-BSA solution. As a result, this could independently or collectively contribute to the observed change in the FITC protolytic state in the FITC-BSA deposits.

DCDR detection sensitivity of FITC-BSA

Detection sensitivity of the FITC conjugated BSA was investigated using 10FITC-BSA. Figure 3 (top) shows a fluorescence image obtained with a series of protein deposits made with 10FITC-BSA solutions and BSA controls on a MALDI plate and (bottom) representative Raman spectra obtained with deposition spot 11, the sample spot that has the least amount of protein (45 fmol) in this experiment. The sampling positions for the Raman spectra (a) and (b) were indicated schematically in the bright field image shown in the inset that clearly shows the preferential protein precipitation at the outer edge of the protein deposits, due to the so-called “coffee-ring” effect.^{18,34} Certainly, for this protein deposit quality, Raman spectra can only be obtained within $5\text{ }\mu\text{m}$ from the very outer edge of the protein ring. No detectable Raman signal can be obtained if the sampling spot moves inward for more than $10\text{ }\mu\text{m}$, as is evidenced by spectrum (b).

For simplicity's sake, assuming that all the protein is uniformly distributed within a μm band of the protein ring with a outer diameter of $\sim 280\text{ }\mu\text{m}$, it can be estimated that for Raman spectra obtained with our 100X objective (NA=0.95), corresponding to a sample size of $2\text{ }\mu\text{m}$ in diameter, the total amount of 10FITC-BSA is about 15 attomole. This sensitivity is significantly higher than the DCDR detection sensitivity reported with label-free proteins.³⁵

FITC Raman signal photobleaching

Even though DCDR exhibits excellent sensitivity with the FITC-labeled protein, its quantitative application is complicated by the surprising FITC Raman and fluorescence photobleaching observed with the 632.8 nm laser. Figure 4 shows the representative DCDR photobleaching data obtained using 100 X objective with (A) 1FITC-BSA and (B) 10FITC-BSA respectively. Data in Fig. 4A depicts the time courses of a FITC Raman peak at 1186 cm^{-1} (black), Raman background intensity calculated around the 1186 cm^{-1} peak (green), and protein Raman peak at 1003 cm^{-1} (blue). The curves in Fig. 4B are the time courses of FITC Raman peak at 1186 cm^{-1} (black) and the Raman background intensity (green). The inset shown in each plot are the first and last DCDR spectra obtained in that dataset obtained for the overnight photobleaching experiment. The red dashed line in each plot was obtained by fitting the time course of the FITC Raman peak at 1186 cm^{-1} with a single exponential function shown as equation 1. The purple dashed curve in Figure 4(A) was obtained by fitting the time course of the fluorescence background intensity of the 1FITC-BSA sample

with two exponential functions since single exponential functions gave large fitting errors. The fitting parameters shown in Table II were obtained with three or four repeated experiments.

$$f(t) = A \exp(-t/\tau) + C$$

Several observations were made with the Raman photobleaching of the FITC-BSA samples. (i) FITC-BSA Raman photobleaching is FITC specific. The Raman spectra in Fig. 4 show that while the FITC Raman features were attenuated with prolonged laser illumination, the BSA Raman features remained essentially intact, indicating that photobleaching can occur at a submolecular level and is limited to the FITC moiety. Such selectivity also suggests that the Raman photobleaching is most likely a result from the chromophore photochemical reactions under laser illumination. To exclude the possibility that photothermal effects of laser illumination cause the FITC Raman photobleaching, DCDR spectra has been obtained with 10FITC-BSA heated at ~120 °C for two hours. The results clearly showed that heat alone doesn't cause a significant change in FITC Raman intensity (data not shown). (ii) Low Raman activity of the photobleached product FITC. Evidently, if the photoreacted product of FITC were Raman active, e.g. with a Raman cross section larger or comparable with BSA, new Raman features would appear in the Raman spectra of 1FITC-BSA, obtained after overnight photobleaching. The total dominance of the BSA Raman feature in the photobleached 1FITC-BSA spectrum shown in the Fig. 4A indicates that compared to BSA, the Raman cross-section of the FITC photoreaction product is significantly smaller. (iii) The curve fitting results shown in Table II indicates that the FITC Raman photobleaching time constants are independent of the chromophore labeling ratios. This is consistent with previous photobleaching results obtained with resonance Raman spectral acquisitions made with intracellular Raman measurements.³⁶

Prior to their photobleaching, the fluorescence background of the DCDR spectra of both 1FITC-BSA and 10FITC-BSA is much higher than that obtained with the label-free BSA sample and the fluorescence background intensity in the FITC-BSA samples scales proportionally with the FITC-labeling ratio (data not shown). These results indicate that FITC is the main, if not the sole contributor to the background fluorescence observed in the FITC-BSA samples. The presence of the FITC fluorescence under our experimental condition is surprising as our 633 nm Raman excitation laser is about 100 nm red-shifted from the red edge of FITC UV absorbance that is centered at the 500 nm region (See Figure 1 for detail). Detailed mechanisms for this off-resonance fluorescence will be discussed later. It is important to note, however, that the FITC background fluorescence at 633 nm is several orders of magnitude smaller than that excited with 488 nm, which explain why FITC Raman spectra can be obtained using 633 nm but not with a 488 nm excitation laser (data not shown). The concurrent appearance of FITC Raman and fluorescence signals in the FITC-BSA Raman spectra, acquired with a HeNe laser, indicates that when excited with 633 nm laser, the FITC fluorescence and Raman cross-section is comparable in magnitude.

One possible mechanism for the FITC fluorescence at 633 nm excitation is two-photon adsorption as FITC-BSA has appreciable UV-vis absorbance in the 316 nm region (See Fig. 1 for detail). With the 100 X objective used in our DCDR spectral acquisition, the laser power density on the sample is in the range of 2MW/cm² to 4 MW/cm². This laser power may in turn make it possible to generate a two-photon excited fluorescence comparable with a FITC Raman signal.³⁷ Experimental results of laser power dependence, however, reveal a linear relationship between the FITC fluorescence background and the laser intensity (Data not shown), indicating that the FITC fluorescence background is generated through a one-photon process.

Unlike FITC Raman photobleaching data for both 10FITC-BSA and 1FITC-BSA, which can be fitted with a single exponential function, the fluorescence photobleaching results in Raman spectra background intensity in the FITC-BSA samples are much more complicated. For the 1FITC-BSA sample, its FITC fluorescence background photobleaching data requires two exponential functions. To our surprise, however, neither of those fluorescence photobleaching time constants, in particular, the one with a large amplitude, resembles the photobleaching constant of FITC Raman photobleaching. Possible explanations include: (a) FITC fluorescence is not among the main contributors to the fluorescence background in the 1FITC-BSA sample. In other words, the bleaching Raman background results from fluorescent impurities unrelated to FITC. (b) FITC is the main contributor to the fluorescence background, but the differences seen in FITC Raman and background photobleaching are indicative that these two processes involve different photobleaching pathways. Clearly, the first explanation is unlikely based on our earlier discussion that FITC-BSA was purified extensively. This would lead us to believe that FITC Raman and fluorescence photobleaching can involve very different mechanisms. One possibility is that fluorescence photobleaching may involve some intermediate states that may be Raman active but not fluorescence active. In fact, fluorescence photobleaching involving nonfluorescent intermediates has been proposed before to explain the multi-exponential fluorescence photobleaching.^{38,39} If these non-emitting intermediates retain their Raman activity and spectral characteristics, one can easily explain why FITC Raman photobleaching is a single exponential with a rate constant much slower than the fast fluorescence decay. It's clear that more theoretical and experimental work is needed to support this hypothesis. As for the 10FITC-BSA sample, the time course of the FITC fluorescence photobleaching is more complicated. Unlike the 1FITC-BSA for which the fluorescence intensity decreases monotonically under continuous laser illumination, the fluorescence background of the 10FITC-BSA increases in the first one hour or so, followed by a monotonic signal reduction with an apparent photobleaching life-time much longer than that for the 1FITC-BSA sample. This result clearly indicates that unlike FITC Raman photobleaching that is independent of its dye labeling ratio, photobleaching of FITC fluorescence is strongly label ratio dependent.

Differential fluorescence and Raman photobleaching characteristics have also been observed in the Raman spectra acquired with 10FITC-BSA solution (without drop drying). Figure 5 shows the photobleaching results obtained with 10FITC-BSA (A) solution Raman spectra and (B) DCDR Raman spectra, both obtained with a 10X objective and 13 mW laser power before objective. While its FITC Raman features remain unchanged during the entire laser illumination period, the fluorescence background of the solution Raman spectra is reduced by more than 20%. In contrast, significant FITC Raman photobleaching was observed in the DCDR spectra of 10FITC-BSA, accompanied with an increasing fluorescence background. Evidently the result in Fig. 5 indicates that both the FITC Raman and fluorescence photobleaching depend critically on the sample condition.

Although the only difference in the DCDR spectra of 10FITC-BSA used for Fig.4 (B) and Fig 5(B) was in their spectral acquisition conditions, their apparent FITC fluorescence and Raman photobleaching features are vastly different. While the FITC fluorescence intensity in Fig.4B increased only in the initial 2500 s of laser illumination and monotonically decreased during the overnight laser illumination, the fluorescence intensity in Fig. 5B increased during the entire 10 hours' laser illumination period. In addition, the photobleaching time constant τ (4254 s) of the FITC Raman signal in Fig.4B is two times smaller than the time constant of 8889 s obtained with the Raman photobleaching data shown in Fig.5B. This result indicates the dependence of FITC fluorescence and Raman photobleaching on the laser power density. The laser power density for the DCDR spectra shown in Fig.4B is ~ 10 times higher (100 X objective with laser power of 1.3 mW) than

that for the DCDR spectra used for Fig.5B (10 X objective with laser power of 13 mW). This result is consistent with the previous reports that the photobleaching rate increases with laser power density.⁴⁰

The increased fluorescence intensity observed in the DCDR spectra of 10FITC-BSA after brief and/or low laser power illumination is very similar to the FITC fluorescence photobleaching profile reported by Hirschfeld.⁴¹ The fact that enhanced fluorescence intensity was only seen in the 10FITC-BSA DCDR spectra, but not in its solution Raman spectra or in the DCDR spectra of 1FITC-BSA, is consistent with the concentration quenching effect proposed by Hirschfeld.⁴¹ Using FITC conjugated globulin and polyethyleneimine, Hirschfeld demonstrated that the FITC fluorescence quantum yield decreases monotonically with increasing label ratio and that the number of effective FITC fluorescence emitters decreases when the dye labeling ratio is larger than a certain threshold value.⁴¹ Consequently, photochemical deactivation of the dye molecule can reduce the dye labeling ratio, leading to an larger number of effective FITC fluorescence emitters and higher fluorescence intensity. Apparently, time courses of the FITC fluorescence shown in Fig.4B and Fig.5B indicate that the FITC packing density in the DCDR deposit of our 10FITC-BSA sample clearly exceeded such a threshold value. As a result, during the initial laser illumination, the packing density of intact FITC decreases, leading to an increased number of effective FITC emitters and a higher FITC fluorescence intensity. However, once the packing density of intact FITC is lower than a certain threshold value where the gain in the fluorescence intensity from the reduced concentration quenching is not sufficient to compensate for the loss of FITC photochemical quenching, continuous laser illumination leads to a monotonically decreased fluorescence.

The differential FITC fluorescence photobleaching characteristics observed in the DCDR and solution Raman spectra of 10 FITC-BSA may also be explained by concentration quenching theory. Although the FITC labeling ratio in these two samples is exactly the same, the intermolecular distance between the FITC labels can be vastly different. Conceivably, due to the low degree of hydration of protein in a DCDR deposit, individual 10FITC-BSA molecules are presumably much more compact. As a result, the intermolecular distance between FITC in a DCDR deposit of 10FITC-BSA is likely to be smaller than that in a 10FITC-BSA solution, which will make the concentration quenching of FITC fluorescence more prominent in a DCDR deposit of 10FITC-BSA.

The independence of Raman photobleaching on the FITC labeling ratio clearly indicates that there is no concentration quenching effect in the FITC Raman signal. Previous research indicates that Raman photobleaching is due to the photochemical reaction of the chromophores at electronically excited states.⁴² To understand the origin of the differential Raman photobleaching characteristics between the 10FITC-BSA solution and its DCDR deposit, UV-Visible spectra of drop-dried 10FITC-BSA was acquired and compared to that obtained with 10FITC-BSA solution. Figure 6 shows that upon drop drying, the FITC UV-Vis peak absorbance was red-shifted from 494 nm in solution to 507 nm and the red-edge of FITC UV-Vis absorption shifted from ~548 to ~574 nm. Detailed reasons for this substantial red-shift are currently unclear, but are likely related to the reduced FITC hydration state and possible FITC aggregation upon drop drying. However, UV-vis data indicates that under a 632.8 nm HeNe Raman excitation condition, it is much more probable to observe pre-resonance or resonance FITC excitation with the drop-dried 10FITC-BSA than its solution sample. This, in combination with the fact that significant Raman photobleaching was observed only in the DCDR spectra of FITC-BSA, but not in its solution Raman spectra, leads us to believe that the FITC photobleaching is due to a photochemical reaction of FITC at the electronically excited states.

CONCLUSION

Normal Raman spectra of FITC-conjugated protein obtained with both solution Raman and the DCDR method are reported here for the first time. With its much higher Raman cross-section, the spectral features from the FITC moiety dominate the spectrum of protein/FITC conjugates, indicating that FITC can be used as a Raman marker for protein detection. Similar to FITC, Raman spectral features of the protein-conjugated FITC is highly pH dependent and the drop drying process used in the DCDR method can modify the protolytic states of protein-conjugated FITC. Using 10FITC-BSA as a model protein, current DCDR detection sensitivity of FITC conjugated protein is 45 fmol in terms of total protein consumption and ~15 attomole at laser probed volume.

The concurrent FITC Raman and fluorescence photobleaching observed in the DCDR spectra of FITC-BSA allowed us to conduct the first comparative study of Raman and fluorescence photobleaching. The FITC Raman photobleaching follows a single exponential decay function with a time constant that has a critical dependence on the sample conditions (solution or DCDR) and excitation laser power density, but not on the FITC labeling ratio. The fluorescence background photobleaching is much more complicated and it has a strong dependence on the FITC labeling ratio, laser power and sample conditions. Mechanistically, the FITC fluorescence photobleaching involves both concentration quenching and photochemical quenching, and the latter may involve a photochemical intermediate that is fluorescence inactive but Raman active. The FITC Raman photobleaching is believed to be primarily due to the photochemical reaction of the FITC molecules in an electronically excited state. Given the fact that fluorescence background and Raman photobleaching are most commonly observed in biological and biochemical Raman application^{40,42-44} understanding their photobleaching mechanisms can be of great importance for correct interpretation of Raman spectral data obtained with such samples.

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Fig. 1. UV-Vis spectra of (a) in-house prepared 1FITC-BSA, (b) commercial 10FITC-BSA and (c) 1FITC-BSA filtrate (cut-off molecular weight: 3500Da). The absence of FITC absorbance at 495 nm in (c) indicates no excess FITC in the purified 1FITC-BSA sample

Fig. 2.
Raman spectra of 10FITC-BSA obtained with (a,c,e) solution and (b,d,f) DCDR at pH's of 9.1(a,b), 7.5(c,d) and 4.9(e,f) respectively.

Fig. 3.

(Top) Fluorescence image of 10FITC-BSA deposits on a MALDI plate. Spots 1, 5 and 9 are the deposits made with 15 μM 10FITC-BSA at deposition volumes of 700 nl, 500 nl and 300 nl, respectively. Spots 2, 6 and 10 are deposits of 1.5 μM 10FITC-BSA at volumes of 700 nl, 500 nl and 300 nl, respectively. Spots 3, 7 and 11 are made with 0.15 μM 10FITC-BSA at depositing volumes of 700 nl, 500 nl and 300 nl, respectively. Spots 4, 8 and 12 are BSA controls with no FITC labeling. (Bottom) Raman spectra obtained of spot 11. The inset photo is the bright field image of spot 11 with the scale bar inside the inset measuring 10 μm .

Fig. 4. Photobleaching curves of DCDR Raman and fluorescence backgrounds for (A) 1FITC-BSA and (B) 10FITC-BSA. The black dot curves in the plots represent the time dependence of the FITC Raman feature at 1186 cm^{-1} , while the green dot curves show the DCDR fluorescence background variation. The blue dot curve in (A) represents the time course for the protein Raman features at 1003 cm^{-1} . Red dot curves were obtained by fitting the FITC Raman photobleaching curve with a single exponential function, while the purple line curve in (A) is obtained by fitting the time course of fluorescence background intensity with two exponential functions. Spectra shown in the inset are (a) the first and (b) the last DCDR spectrum obtained during the overnight photobleaching experiment. All the spectra used in

this figure were acquired with a 100X objective and with 1.3 mW laser power before objective.

Fig. 5. Photobleaching curves of (A) 10FITC-BSA solution and (B) 10FITC-BSA DCDR deposit. The black and green dot curves represent the time course of the FITC Raman feature at 1186 cm^{-1} and fluorescence background feature respectively. The red dashed line was obtained by fitting the FITC Raman photobleaching data with a single exponential decay. Both the DCDR and solution Raman spectra used in this figure were acquired with a 10X objective and with 13 mW laser power before objective.

Fig. 6. The UV-Visible spectrum of (a) 10 FITC-BSA solution, and (b) drop dried deposit of 10FITC-BSA on quartz slide. The inset shows the red-edge of the UV-Vis spectra of the 10FITC-BSA.

Table I

The assignments of DCDR and solution Raman vibrational bands (cm^{-1}) observed at basic, neutral and acidic conditions for FITC-BSA conjugates

pH 10	pH 7.7	pH 4.9	Assignments
764	764	764	Xanthene ring deformation ⁴⁵
1171			Phenolic OH ⁴⁶
	1186	1186	Phenolic OH ⁴⁶
1313	1313 ^a		Phenoxide ion stretch conjugated with
			Xanthene ring stretching ⁴⁷
1334 ^a	1334	1334	Xanthene C-C stretch ³³
1417	1417	1417	
1505	1505	1505	In plane ring vibrations (?)
	1562	1562	Xanthene ring C-C stretching ^{33,45}
1604	1604	1604	Xanthene C-C with conjugated carbonyl band ⁴⁸
1637	1637	1637	Xanthene ring C-C stretching ³³

^a Observed only in solution spectra

Parameters obtained by fitting the FITC Raman intensity at 1186 cm^{-1} and fluorescence background photobleaching data with one and two exponential functions, respectively.

Table II

Sample	Spectral features	A1	τ_1 (s)	A2	τ_2 (s)
1FITC-BSA	FITC Raman	1561 ± 151	4249 ± 511		
	Fluorescence Background	23502 ± 1236	421 ± 176	7475 ± 320	5483 ± 833
10FITC-BSA	FITC Raman	12011 ± 1461	4254 ± 973		