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High-sensitive Raman measurements of protein aqueous solutions using liquid-liquid phase separation†

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A highly sensitive method is proposed for obtaining Raman spectra of low-concentration proteins and nucleic acids in an aqueous solution using liquid-liquid phase separation. This method uses water droplets formed by adding a large amount of polyethylene glycol into a biomolecular aqueous solution. Ordinary spontaneous Raman spectra are obtained with a high signal-to-noise ratio.

Raman spectroscopy, one of the vibrational spectroscopic methods, has long been used to analyse structures of biomolecules in aqueous solution.[1–4] The advantage of Raman spectroscopy in biology is its ability to perform measurements of molecules in aqueous solutions, where infrared (IR) measurements are difficult due to the strong IR absorption of water. The disadvantage of Raman spectroscopy is that the Raman signal is weak, and measurements of proteins in aqueous solution usually require a high concentration of 1 mM or more. This problem has not yet been solved and hinders its application to various biological samples. Protein measurements are hence sometimes carried out in solid and dense states, where a protein solution is air-dried. High-sensitive Raman detections can be performed using surface-enhanced Raman scattering (SERS). In general, however, both the intensity and spectral shape of a SERS spectrum markedly change with slight changes in SERS-inducing nanostructures, making quantitative spectral analyses difficult.

This study proposes a high-sensitive Raman measurement method for biomolecules such as proteins in aqueous solutions using liquid-liquid phase separation (LLPS). LLPS in biology is water-water phase separation in which an aqueous solution of a biomolecule is divided into a high-concentration aqueous phase (droplet) and a low-concentration aqueous phase.[5–8] LLPS has extensively been studied in biology, which comes from the fact that many biological phenomena such as gene expression and the onset of neurodegenerative diseases are explained by the occurrence of LLPS.[9,10] LLPS also occurs in highly concentrated polyethylene glycol (PEG) aqueous solutions containing buffer molecules such as phosphate ions; droplets containing only water and buffer molecules are produced in PEG solutions.[11–14] This study shows that by condensing proteins in water droplets in a high-concentration PEG solution, Raman spectra of proteins at a concentration as low as 30 μM can be measured with a high signal-to-noise (S/N) ratio. Various proteins and nucleic acids are concentrated to 1 mM or higher in water droplets in a high-concentration PEG solution, and applications such as the detection of denaturation and supersulphidation are presented.

We dissolved PEG powder into a diluted (10–50 μM) protein buffer solution, resulting in a final concentration of PEG of ~50 wt%. Then we gently stirred the mixed solution, succeeding in the selective protein condensation in water droplets (Fig. S1 in ESI†). Fig. 1 shows the images of a single water droplet including various proteins (human serum albumin (HSA), superoxide dismutase 1 (SOD1), enhanced green fluorescent protein (EGFP), cytochrome *c* and myoglobin) formed in the PEG

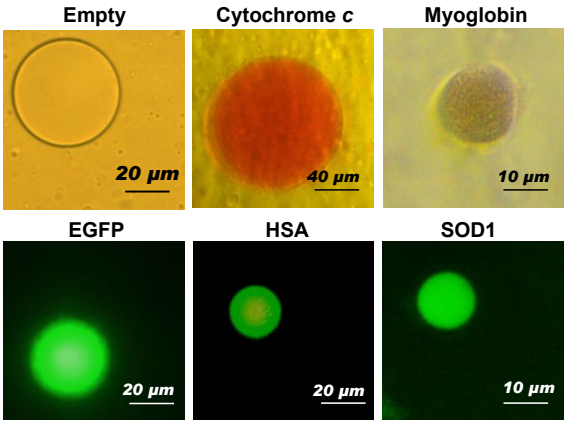

Fig. 1 Examples of a protein-encapsulated droplet. Cytochrome *c*, myoglobin, EGFP, HSA and SOD1 were concentrated in a water droplet formed by a high-concentration PEG aqueous solution. Bright-field images are shown for droplets containing cytochrome *c* and myoglobin. EGFP, HSA and SOD1 were fluorescent-labelled, and droplets containing them were observed by a fluorescence microscope. An empty droplet containing no protein is also shown at the upper left.

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solution together with a water droplet without proteins. Proteins exhibiting no visible absorption or fluorescence were labelled with a fluorescent dye. The protein condensation in water droplets can be confirmed by the marked difference in colour between the inside and outside of the droplets. The protein-encapsulated droplets are stable, and no protein leakage is observed for at least 1 h. Protein-PEG solutions produce both protein-encapsulated and non-encapsulated droplets, the ratio of which depends on the concentration of the initial protein solution and the protein used. The formation of protein droplets using high-concentration PEG is hereafter called the LLPS method.

It is already known that protein droplets are formed using PEG and phosphate buffer.[14] We systematically varied the concentrations of phosphate ions, NaCl and PEG to examine the conditions for forming protein droplets. In this study, the phosphate ions, NaCl and PEG concentrations were set to 50 mM, 300-400 mM and 50 wt%, respectively, to efficiently induce the droplet formation (Fig. S2 in ESI†). A PEG concentration below 50 wt% made it difficult to form droplets, and PEG could not be completely dissolved in water at concentrations over 70 wt%. The average molecular weight of PEG was 6000, and it was difficult to prepare water droplets using PEG with lower average molecular weights. Slow stirring for approximately 5–10 minutes was essential for forming protein droplets. The proteins in Fig. 1 intrinsically have a low ability to form their LLPS droplets. It is thus appropriate to consider that the protein droplets prepared in this study were formed by trapping proteins inside water droplets present in a highly concentrated PEG solution.

Raman spectra inside a single droplet were measured using a confocal inverted Raman microscope, and the results are shown in Fig. 2. The Raman spectrum of a 30-μM dispersed protein solution is also shown in the same figure. The measurement time was 60 s, the excitation light was 532 nm, and the laser intensity at the entrance of the objective lens was ~45 mW. In a 30-μM protein aqueous solution, only Raman bands of water were observed due to the low protein concentration. However, many Raman bands were observed when the single protein droplets produced by the LLPS method were focused under a microscope. All the proteins in Fig. 2 exhibit the Raman spectra with a high S/N ratio, and their spectral shapes are different from the Raman spectrum of PEG observed outside the droplets (Fig. S3 in ESI†). The Raman bands of the droplets are almost assignable to the proteins. Actually, the Raman spectrum of the dispersed protein solution at ~3 mM has almost the same shape and peak positions as that of the droplets (Fig. S4 in ESI†). The solution is highly viscous, so the droplets are almost motionless, facilitating Raman measurements using an inverted microscope. All these results indicate that the high-sensitive Raman measurements of proteins in a diluted aqueous solution were successfully performed using the LLPS method.

Strong Raman bands of PEG were observed on the outside of droplets, whereas there were no or very small Raman bands of PEG inside the empty droplets with no protein encapsulated. (Fig. S3 in ESI†). This result indicates that PEG does not enter droplets regardless of the presence or absence of proteins. Raman bands of phosphate ions, the buffer molecule, were strongly observed in empty droplets, and phosphate ions were concentrated more than 100 times inside empty droplets (Fig. S3 in ESI†). In protein droplets, however, the Raman intensity of phosphate ions as well as PEG was small, suggesting that the incorporation of proteins into droplets causes the exclusion of buffer molecules inside droplets. Thus, the contribution of phosphate and PEG to the spectra of protein droplets can be negligible or easily removed by subtracting the spectra of PEG and phosphate ions.

The highly sensitive protein observations arise from the pronounced protein condensation in water droplets. We thus quantified the protein concentration in droplets. The O-H stretching band at around 3500 cm^{-1} of water outside can be used as an intensity standard for quantifying the Raman intensity.[15–17] This is because the outside of droplets is dominated by water, and the concentration of water molecules in water outside can be regarded as a constant regardless of the sample. It is noted here that the O-H stretching band of water is very strong, allowing for a highly accurate correction of

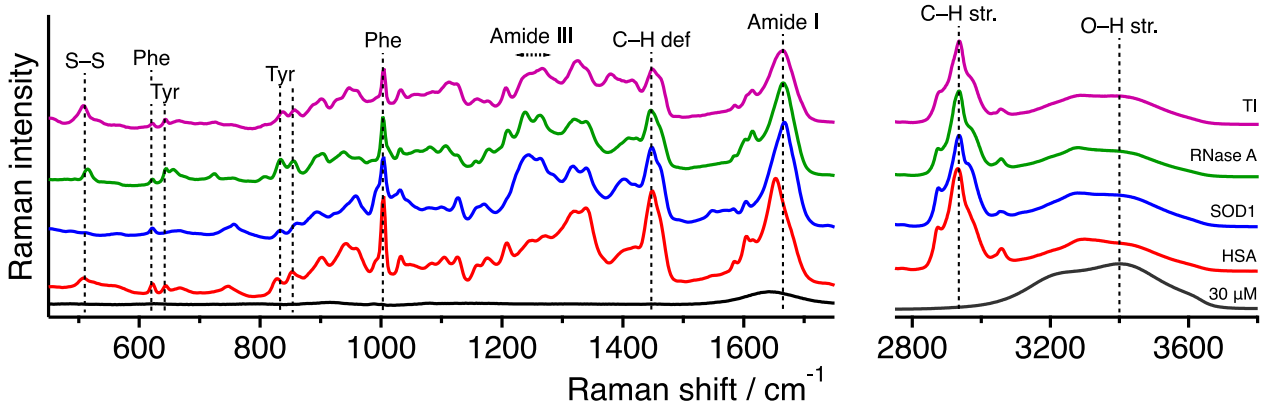

Fig. 2 Raman spectra of a protein droplet of TI (trypsin inhibitor) (purple), RNase A (green), SOD1 (blue), HSA (red) in a high-concentration PEG solution, together with a dispersed solution of HSA at 30 μM. The Raman spectra of protein droplets were roughly normalised to the O-H stretching band in the region at around 3500 cm^{-1}. The excitation wavelength was 532 nm.

experiment-to-experiment variations in the laser intensity and optical alignment. We measured Raman spectra of protein dispersion solutions at various concentrations to make a calibration line between the protein concentration and the intensity of the amide I band of the protein at around 1680 cm^{-1} relative to that of the O-H stretching band. Then, the intensity of the amide I band in the droplet relative to that of the O-H stretching band outside was calculated and compared to the calibration line to obtain the protein concentration in the droplet (Fig. S5 in ESI†). For HSA, the concentration obtained was 13–17 mM, meaning that it is more than 100 times more concentrated than the original dispersed solution at 30 µM. The obtained concentration is comparable to the reported concentration of the droplets of the proteins intrinsically having a LLPS ability.[15,16] The concentration inside the droplet did not substantially change when the concentration of the initial diluted protein solution was varied (Fig. S5 in ESI†). This result suggests that reducing the concentration of the initial protein solution does not reduce the internal concentration but rather reduces the proportion of protein-encapsulating droplets among the droplets existing in a PEG aqueous solution. We previously studied the protein concentration inside droplets of a protein having the ability to exhibit LLPS[16] and found that it was almost insensitive to the initial protein concentration. The present result is in line with our previous finding.

The present high-sensitive Raman measurement can be performed simply by adding PEG into a protein solution. A 50-µL of protein solution is sufficient for droplet formation and subsequent Raman measurements. The required protein concentration depends on the protein, and the protein solutions used in this study were 10-50 µM. Protein droplets can also be made using carbonate ions instead of phosphate ions as a buffer molecule, and Raman measurements were successfully performed (Fig. S6 in ESI†). The obtained spectral shape was the same as that in a phosphate buffer. Carbonate ions were concentrated in the droplet in the absence of a protein, while the condensation of carbonate ions was suppressed in the presence of a protein, as was the case of phosphate ions. Acetic acid and dextran were also used to prepare protein-encapsulated droplets (Fig 3); however, a ~100 µM protein solution seems to be necessary to prepare a sufficient amount of protein-encapsulated droplets. The Raman spectrum of cytochrome *c* droplets could not be observed with a high S/N ratio. This is because cytochrome *c* has an absorption at around 530 nm,[18] and the absorption of the excitation light causes weak fluorescence and a temperature increase inside the droplet, resulting in protein aggregation and degradation. Thus, resonance Raman measurements may be difficult to achieve with this method.

The LLPS method can be used to detect structural changes in proteins. After denaturing HSA with high-temperature treatment, HSA was concentrated into water droplets. Raman spectra of the droplets of HSA treated at elevated temperatures showed a high wavenumber shift of the amide I band, indicating the detection of the structural change to random and ß-sheet structures using the LLPS method (Fig. S7 in ESI†). We also applied the LLPS method to detect supersulphidation in a protein. Supersulphide structures are those in which excess sulphur atoms are covalently bonded to a thiol group or a disulphide bond in a biomolecule.[19,20] Raman spectra of the droplets of insulin and RNase A after the treatment with Na_2S_4 are shown in Fig. 3 and Fig. S8 in ESI†, respectively. Treating proteins with Na_2S_4 is expected to extend S-S bonds in proteins into trisulphides, tetrasulphides, etc..[21,22] The position of the S-S stretching band at 500 cm^{-1} varies with the catenation of a sulphur atom; disulphide bonds show the S-S stretching band above 500 cm^{-1} while supersulphide structures such as trisulphides show lower wavenumber shifts of the S-S band below 500 cm^{-1}.[23,24] The Raman spectra of insulin (Fig. 3) and RNase A (Fig. S8 in ESI†) treated with Na_2S_4 exhibited an increased intensity in the region at around 490 cm^{-1}, indicating the presence of supersulphide structures in these proteins. It has recently been reported that supersulphide structures are present in mammalian cells and regulate various physiological phenomena.[25] The present LLPS method can detect supersulphide structures using a 50-µL diluted protein solution,

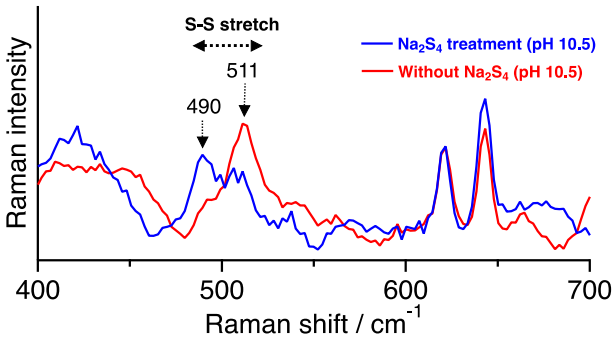

Fig. 3 Raman spectra of a protein droplet of insulin with (blue) and without (red) Na_2S_4 treatment at pH 10.5 in a high-concentration dextran solution. The Raman intensity was normalised by the sharp band due to phenylalanine at 620 cm^{-1}. The Na_2S_4 treatment increased and decreased the Raman intensity at around 490 and 511 cm^{-1}, respectively, indicating an increase in the supersulphide structures. The excitation wavelength was 532 nm.

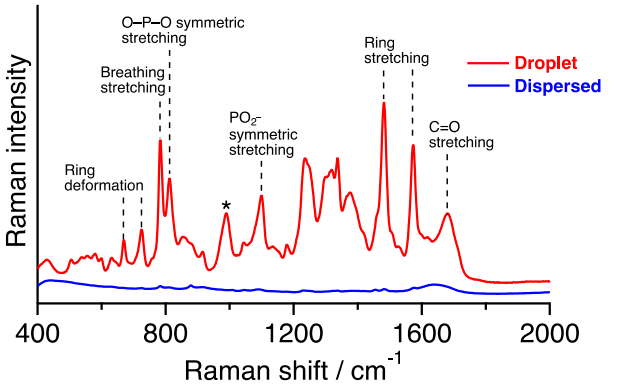

Fig. 4 Raman spectra of RNA inside a water droplet in the PEG solution (red) and in a dispersed solution (blue). The concentration of RNA was 5 mg/mL (red) and 10 mg/mL (blue). The Raman spectra were roughly normalised to the O-H stretching band of water at around 35□□ 00cm^{-1}. The asterisk band arises from the concentrated phosphoric acids in the droplets. The excitation wavelength was 532 nm.

and it is further expected to identify the detailed supersulphide structure from the peak position of the S-S Raman band.

The LLPS method is expected to be applicable to a variety of biopolymers. We successfully concentrated RNA in water droplets in a high-concentration PEG solution and obtained Raman spectra of RNA in the droplets with a high S/N ratio (Fig. 4). The Raman bands due to ring stretching and deformation of pyrimidines of RNA were clearly observed, and such Raman bands are difficult to be observed at similar concentrations in dispersed solutions. We also concentrated an antibody (γ-globulin) in water droplets and obtained a Raman spectrum with a high S/N ratio (Fig. S9 in ESI†). The Raman spectrum of the antibody was successfully measured even at very low concentrations of 100 nM. The Raman spectrum of the antibody in the droplets is the same as that in the dispersed state, and the present LLPS method is expected to be used to confirm the binding of a protein, such as an antibody with substrates.

In our experiments, droplets were not formed without buffer molecules. In addition, not all proteins can be concentrated into droplets in highly concentrated PEG solutions. Proteins with low water solubility or small size seem to have difficulty concentrating in the droplet. To apply the LLPS method to various systems, it is necessary to expand the range of biomolecules that can be concentrated in droplets using other buffers and polymers.

In conclusion, we have proposed a high-sensitive Raman method for diluted protein or RNA aqueous solutions using LLPS. This method is simple, as it only involves dissolving a high concentration of PEG in an aqueous biomolecule solution. Measurements can be performed at low concentrations of 10-50 μM and with small volumes of about 50 μL, and the ordinary spontaneous Raman spectra are obtained with a high S/N ratio, allowing detailed spectral analyses. The present method is expected to be applied to other Raman spectroscopic techniques such as non-linear Raman and Raman optical activity.[26]

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

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Data Availability Statement

The data supporting this article have been included as part of the Supplementary Information.