

## Electronic Supplementary Material (ESI)

# Highly sensitive Raman measurements of protein aqueous solutions using liquid-liquid phase separation

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## 1. Experimental Procedures

**Samples.** The following chemicals were purchased from Nacalai Tesque, Japan: sodium chloride (NaCl) (09649-15), sodium dihydrogen phosphate dihydrate (31717-25), disodium hydrogen phosphate 12-water (31723-35), Sodium carbonate (31311-25), polyethylene glycol #6,000 (PEG) (28254-85), trypsin inhibitor (35546-61), human serum albumin (HSA) (19597-01), ribonuclease A (RNase A) (30100-31), insulin (human, recombinant expressed in yeast, animal-free) (12878-44). Cytochrome *c* from bovine heart (C2037) and myoglobin from equine skeletal muscle (M0630) were purchased from Sigma-Aldrich. Magnesium Sulfate (Anhydrous) (137-12335), Dextran 200000 (041-22612) and  $\gamma$ -globulin (075-06691) were purchased from Fujifilm Wako, Japan. *N*-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) (GB06) and sodium tetrasulfide (SB04) were purchased from Dojindo, Japan. All the chemicals purchased were used as received. Cu, Zn superoxide dismutase (SOD1) was prepared as described in our previous papers.<sup>1,2</sup> EGFP prepared at Hokkaido University, Japan, was used.<sup>3</sup>

SOD1 and HSA were labelled using Alexa Fluor 488 Protein Labelling Kit (Thermo Fisher Scientific) for fluorescence imaging. Briefly, 50  $\mu$ L of a 1 M bicarbonate buffer was added to 200  $\mu$ L of a 2 mg/ml HSA solution or a 125  $\mu$ M SOD1 solution. The solutions were then transferred to a reaction vial containing Alexa Fluor 488 NHS ester, and the labelling reaction was carried out for 1 h at room temperature. The dye molecules that did not bind to the proteins were removed using a centrifugal filter (Amicon Ultra-0.5 mL, 3000 MWCO, Merck Millipore) or a purification column (included in the kit). Successful labelling was confirmed by measuring the absorption spectra of the resulting protein solutions.

**Preparation of droplets.** All the experiments were performed at room temperature. Droplet formation in high-concentrated PEG aqueous solutions was mainly carried out using phosphate buffer. A phosphate buffer solution of a biopolymer was prepared with a concentration of 0.1-50  $\mu$ M biopolymer, 300-400 mM sodium chloride, 50 mM phosphoric acid at pH 7.4 and a total volume of 100  $\mu$ L. 100 mg of PEG6000 was weighed to make a 50 wt% solution, added to the prepared solution and dissolved with a stir bar. The mixture was stirred gently at approximately 50 rpm for 5–10 minutes, and after visually confirming that PEG6000 was completely dissolved, 20  $\mu$ L of the PEG solution was dropped into a glass bottom dish (Matsunami) for measurements.

Droplet formation in carbonate buffer was performed using a 0.1-50  $\mu$ M biopolymer solution, 400 mM sodium chloride, 50 mM carbonate ions at pH 10 and a total volume of 100  $\mu$ L. 100 mg of PEG6000 was added to the prepared solution and dissolved with a stir bar (ca. 50 rpm) for 5–10 minutes. After visual confirmation that PEG6000 was wholly dissolved, 20  $\mu$ L of the prepared solution was dropped into a glass bottom dish for measurements.

Droplet formation in 30% acetic acid was performed using a 100  $\mu$ M protein solution, 600 mM  $\text{MgSO}_4$  at pH 1.5~2 and a total volume of 300  $\mu$ L. 300 mg of dextran was added to the prepared solution and dissolved with a stir bar (ca. 50 rpm) for 5–10 minutes. After visual confirmation that dextran was wholly dissolved, 50  $\mu$ L of the prepared solution was dropped into a glass bottom dish for measurements.

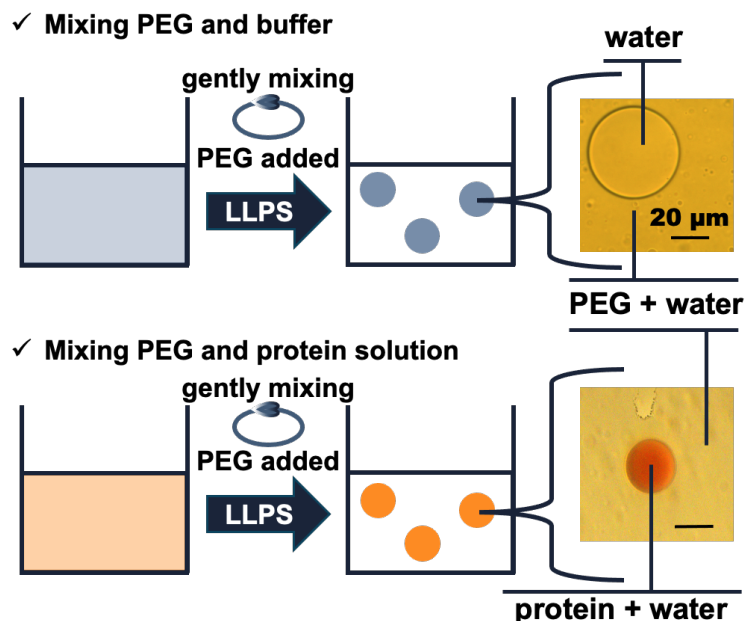
**Denaturation.** HSA was dissolved in phosphate buffer (50 mM, pH 7.4) to 50  $\mu$ M and incubated in a water bath at 80 °C for 10 min. Denaturation was confirmed by a circular dichroism (CD) spectrum using a CD spectrometer (JASCO).

**Supersulphidation.** Insulin was dissolved in CAPS buffer (50 mM, pH 10.5) to 3.3 mg/mL. 20 mM  $\text{Na}_2\text{S}_4$  solution was prepared by adding 3.5 mg  $\text{Na}_2\text{S}_4$  in 1 mL of CAPS buffer. Then 900  $\mu$ L of the insulin solution and 100  $\mu$ L of the  $\text{Na}_2\text{S}_4$  solution were mixed so that the final concentration of insulin and  $\text{Na}_2\text{S}_4$  would be 3 mg/mL and 2 mM, respectively. The mixed solution was incubated at 37 °C for 30 min in a light-shielded environment. After the incubation, the solution was introduced onto a size-exclusion chromatography column (PD-10, Cytiva) and NaOH solution (0.01 M) was run through the column to remove  $\text{Na}_2\text{S}_4$  and CAPS.

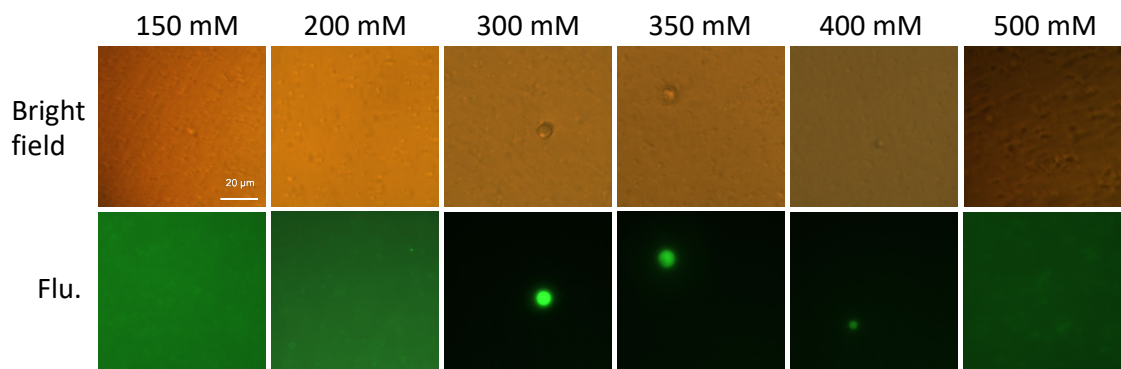
RNase A was dissolved in CAPS buffer (100 mM, pH 10) to 200  $\mu$ M. 200 mM  $\text{Na}_2\text{S}_4$  solution was prepared by adding 3.5 mg  $\text{Na}_2\text{S}_4$  in 100  $\mu$ L of milli-Q. Then 250  $\mu$ L of the RNase A solution, 100  $\mu$ L of the  $\text{Na}_2\text{S}_4$  solution and 150  $\mu$ L of CAPS buffer were mixed so that the final concentration of RNase A and  $\text{Na}_2\text{S}_4$  would be 100  $\mu$ M and 40 mM, respectively. The mixed solution was incubated at 37 °C for 60 min in a light-shielded environment. After the incubation,  $\text{Na}_2\text{S}_4$  was removed using a size-exclusion chromatography column, and the buffer of the protein solution was exchanged to phosphate buffer.

**Measurements of confocal Raman spectra.** Raman spectra were measured using a homemade inverted confocal Raman microscope.<sup>4</sup> 20  $\mu$ L of an aqueous protein-PEG solution was dropped into a glass-bottom dish (Matsunami), and the dish was covered to prevent evaporation and placed on an inverted microscope (IX-73, Olympus). The beam from a CW frequency-doubled Nd:YVO<sub>4</sub> laser (Millennia Vs, Spectra-Physics) was used for the 532-nm excitation and that from a CW Ti:sapphire laser (3900S, Spectra-Physics) pumped by the frequency-doubled Nd:YVO<sub>4</sub> laser was used for the 785-nm excitation. The excitation beam was focused on a sample through a 100 $\times$  (oil immersion) or 60 $\times$  (water immersion) objective (Olympus). The Raman signal passing through a pinhole providing a confocal condition was dispersed by a polychromator (SOL instruments, MS3504) and detected by a thermoelectric-cooled CCD (DV420A-OE, Andor).

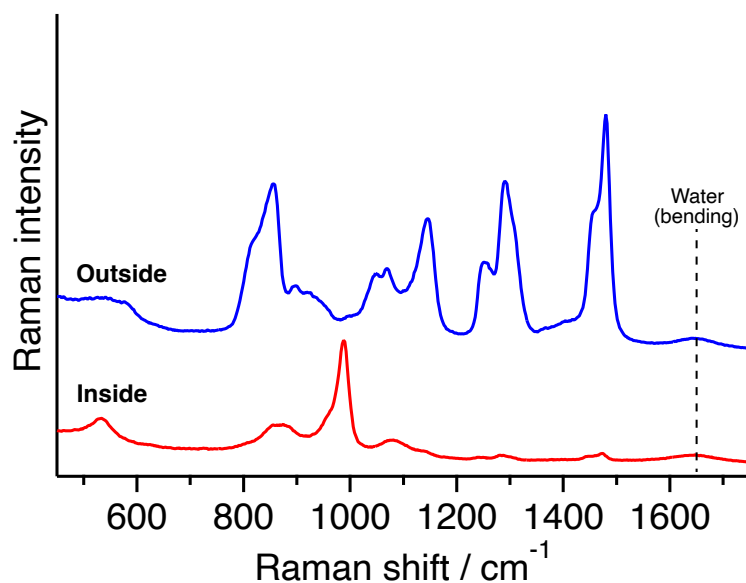
## 2. Figures



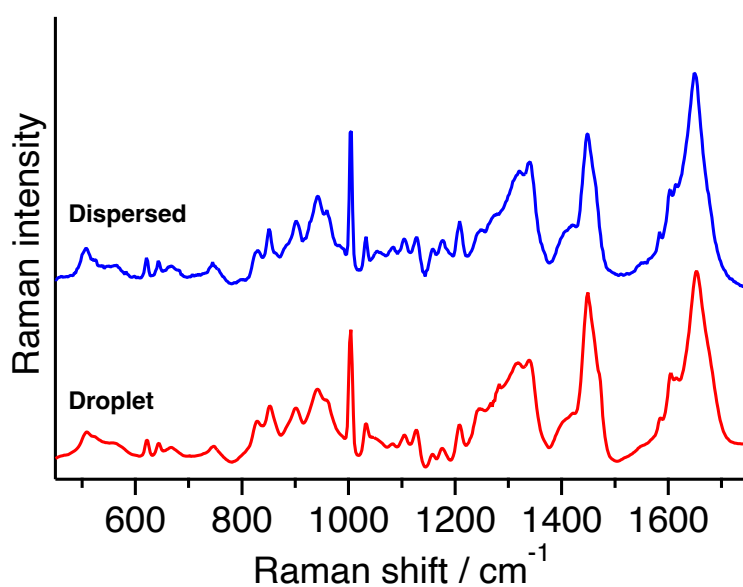
**Fig. S1** Conceptual diagram of how to concentrate proteins into water droplets in a high-concentration PEG solution.



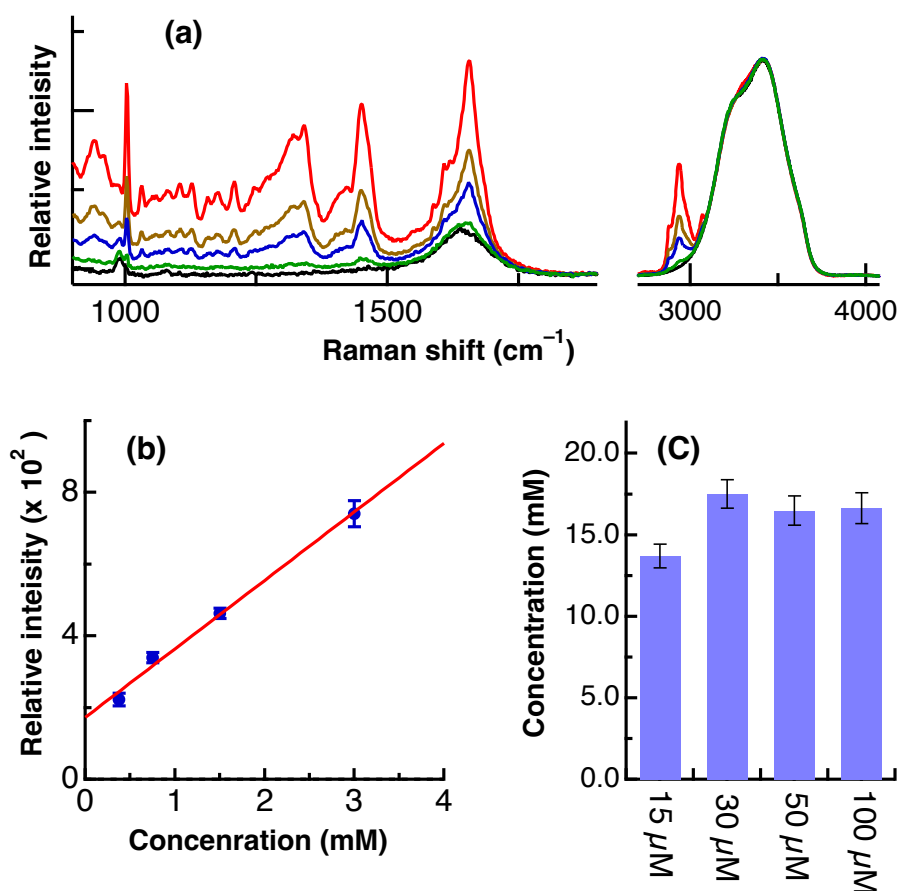
**Fig. S2** Salt-concentration dependence of bright field (top) and fluorescence (bottom) images of an EGFP-PEG aqueous solution. The salt concentration of each image set is indicated above. EGFP-incorporated droplets showing strong fluorescence appear at 300-400 mM salt concentrations. The concentrations of PEG and EGFP were 50 wt% and 10 μM, respectively.



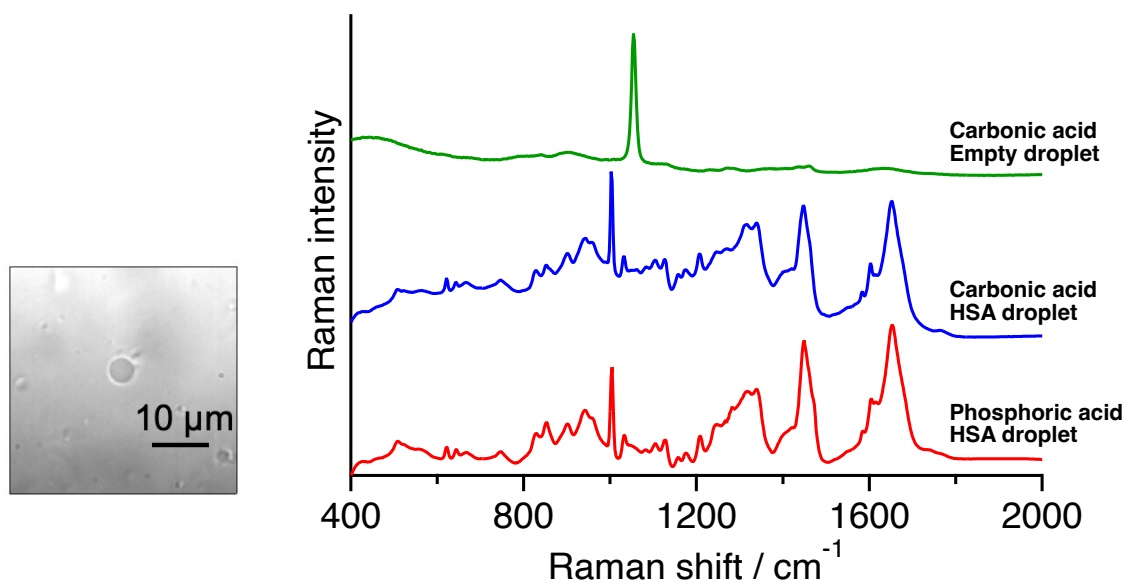
**Fig. S3** Raman spectra of the outside (blue) and inside (red) of the empty droplets formed in phosphate buffer without encapsulated proteins. The Raman spectra were roughly normalised to the O-H stretching band of water at around  $3500\text{cm}^{-1}$ . The observed bands are assigned to PEG (outside) and phosphoric acids (inside). Small (low-intensity) bands of PEG on the inside Raman spectrum are due to the outer PEG being reflected in the spectrum due to low resolution along the Z-axis. The excitation wavelength was 532 nm.



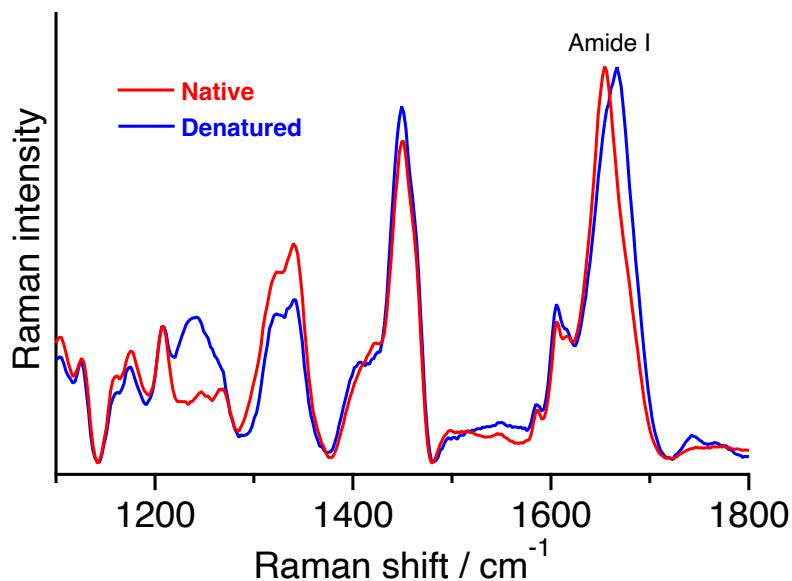
**Fig. S4** Comparison of the Raman spectra of HSA as a dispersed solution (blue) and a single droplet (red). The Raman spectra were roughly normalised to the amide I band at around  $1650\text{ cm}^{-1}$ . The concentration of the dispersed solution was  $\sim 3\text{ mM}$ . The spectral shape is almost the same between these two spectra. The excitation wavelength was 532 nm.



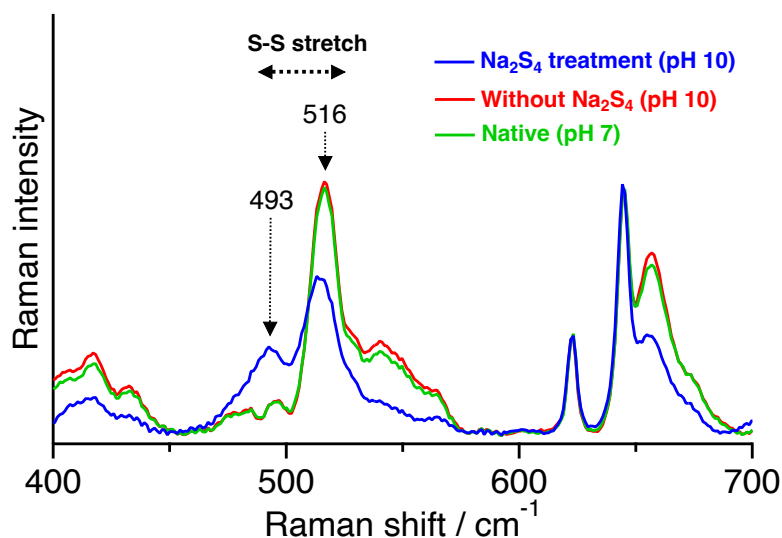
**Fig. S5** (a) Raman spectra of HSA in dispersed aqueous solutions with different concentrations. Green; 0.38 mM, blue; 0.75 mM, Yellow; 1.5 mM, Red; 3.0 mM. The Raman intensity at each spectrum was roughly normalised to the O-H stretching band of water at around  $3500 \text{ cm}^{-1}$ . (b) Plots of the Raman intensity of the amide I band relative to that of the O-H stretching band of water against the HSA concentration. The integrated intensities in the regions of  $1620\text{--}1700 \text{ cm}^{-1}$  and  $3500\text{--}3600 \text{ cm}^{-1}$  were used as the intensity of the amide I and O-H stretching bands, respectively. The intensity of the bending band of water around  $1600 \text{ cm}^{-1}$  was not subtracted in evaluating the amide I band. Standard error was used;  $n = 3$  at each spectrum. (c) Initial concentration dependence of the HSA concentration in water droplets obtained from the calibration line in (b). Standard error was used;  $n = 6$  (15  $\mu\text{M}$ ),  $n = 15$  (30  $\mu\text{M}$ ),  $n = 16$  (50  $\mu\text{M}$ ),  $n = 14$  (100  $\mu\text{M}$ ). The excitation wavelength was 532 nm.



**Fig. S6** (Left) An image of an HSA droplet formed by adding PEG into an HSA solution with carbonic acid buffer. (Right) Raman spectra of the droplets without encapsulated proteins in PEG-carbonic acid buffer (green), HSA droplets in PEG-carbonic acid (blue) and PEG-phosphoric acid (red) buffer. The concentration of HSA was 30 μM. The Raman spectra were roughly normalised to the O-H stretching band of water at around 3500cm<sup>-1</sup>. The red spectrum is the same as that in Fig. S4. The excitation wavelength was 532 nm.

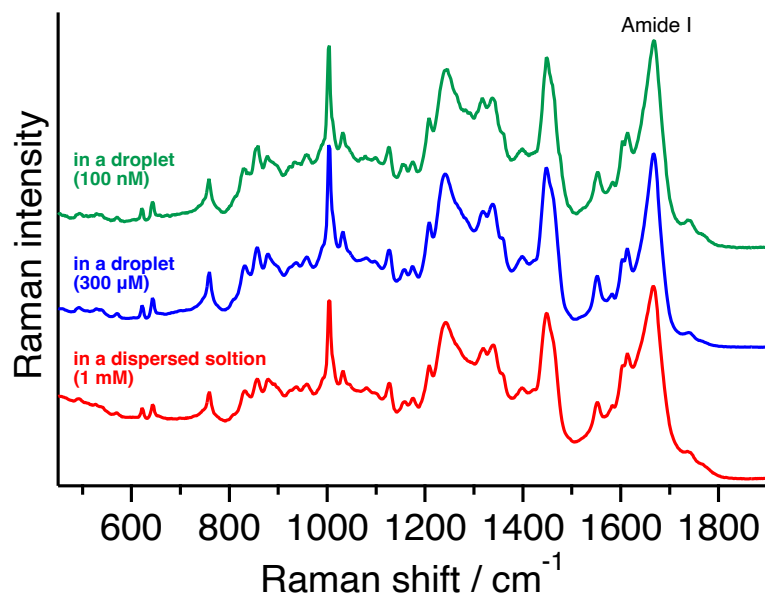


**Fig. S7** Comparison of the Raman spectra of HSA in the native state (red) and after high-temperature treatment (blue) using the LLPS method. The concentration of HSA was 30  $\mu\text{M}$  in both cases. The high-wavenumber shift of the amide I band was observed for the high-temperature-treated HSA, which is due to the change to random and  $\beta$ -sheet structures caused by denaturation. The excitation wavelength was 532 nm.



**Fig. S8** Raman spectra of RNase A with (blue) and without (red) Na<sub>2</sub>S<sub>4</sub> treatment at pH 10. The Raman spectrum of RNase A at pH 7 is also shown for comparison (green). The Raman intensity was normalised by the sharp band due to phenylalanine at 620  $\text{cm}^{-1}$ . The Na<sub>2</sub>S<sub>4</sub> treatment increased and decreased the Raman intensity around 493 and 516  $\text{cm}^{-1}$ , respectively, indicating an increase in the supersulphide structures. The excitation wavelength was 785 nm.





**Fig. S9** Comparison of the Raman spectra of antibody (immunoglobulin) in a water droplet in a PEG aqueous solution (blue and green) and in a dispersed aqueous solution (red). The concentrations of the prepared solutions were 100 nM (green), 300  $\mu\text{M}$  (blue) for the droplets and 1 mM for the dispersed solution. The intensity was roughly normalised to the amide I band. The excitation wavelength was 532 nm.

### 3. References

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3. T. Nakabayashi, H.-P. Wang, M. Kinjo and N. Ohta, *Photochem. Photobiol. Sci.*, 2008, **7**, 668-670.
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