Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Supporting Information

Micro-Raman Spectroscopic Analysis of Liquid–Liquid Phase Separation

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Supplementary Method. Depth scanning

Z-scan of micro-Raman experiments was repeated using two imaging spacers with different depths (120 and 6 μm each) to estimate the thickness of the bulk water layer below the droplet in the depth of the focus region. We hypothesized that the Raman intensity of the O-H stretching band is increased when the focal point is moved below the droplet if the bulk water layer under the droplet is dominant in the region of the depth of focus. To test the hypothesis, we set Z equal to 0 at the point where the Raman intensity of the amide I band of protein is the greatest. Next, we monitored the intensity change of the amide I and the O-H stretching band by moving along the z-axis within the distance range of 10-100 μm at the same lateral position. When using the 120 μm imaging spacer (Figure S2c), the intensity of the O-H stretching band increased as the focal point moved down 10 μm from the sample stage set as Z=0. This indicates that the bulk water layer below the droplet is dominant in the depth of focus at the point, Z=10 μm. Meanwhile, the intensity of the O-H stretching band decreased as the focal point moved down when using the 6 μm Teflon spacer (Figure S2d), which means the bulk water layer under the droplet is negligible. Therefore, we used 6 μm spacers because Raman spectra of the sample with 6 μm spacers better describe the structure inside the droplet, considering that the bulk water layer can be neglected within the depth of focus.

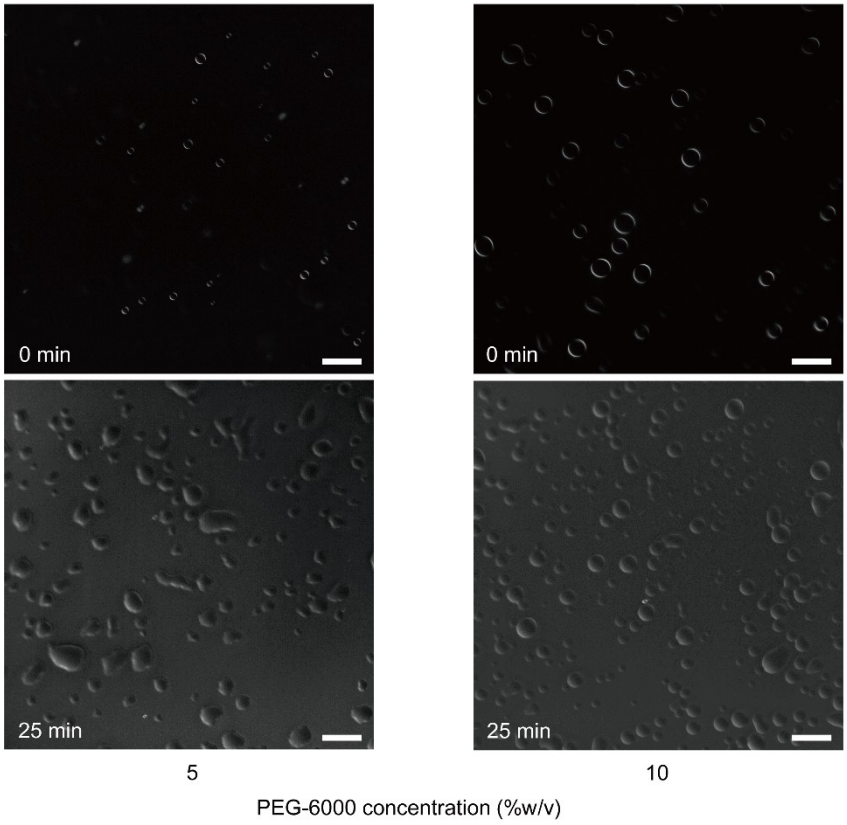

Figure S1. Wetting property of liquid droplets of Lys−Alb heteroprotein mixture

DIC images showing a wetting property of liquid droplets when encountering the surface of a coverslip. Time refers to imaging time. Imaging spacers (120 µm) were used and the exposure time was set to 0.1 s/pixel. Scale bar: 10 µm. The droplets with 5% w/v PEG more rapidly wetted the glass surface than droplets with 10% w/v PEG. We found that the shape of spherical droplets became irregular as they were adsorbed onto the glass surface.

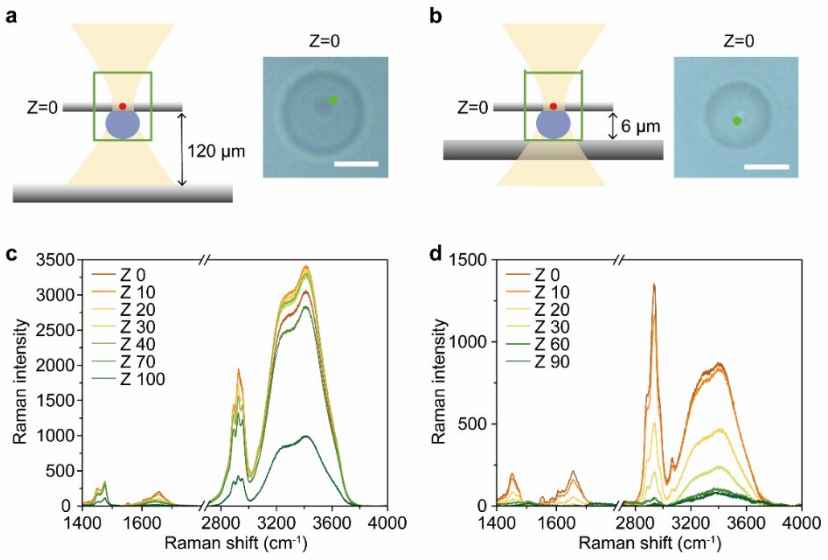

Figure S2. Analysis of the effect of the depth of imaging spacer on spectral features

(a), (b) Cartoons represent depth scanning of micro-Raman experiments using 120 µm and 6 µm spacers, respectively. Bright-field images indicate the liquid droplet at the point Z=0, where the intensity of the amide I band is maximized. To minimize the influence of the liquid/solid interface, only spherical droplets were selected and measured. (c), (d) Raman spectra measured within the droplet corresponding to the Z-scanning using 120 µm and 6 µm spacers, respectively. Z-scan of Raman spectra were taken at depths of 10, 20, 30, 40, 70, 100 µm using 120 µm spacer, and at depths of 10, 20, 30, 60, 90 µm using 6 µm spacer. When using the 120 µm imaging spacer, the intensity of the O-H stretching band increased as the focal point moved down 10 µm from the sample stage set as Z=0. This indicates that the bulk water layer below the droplet is dominant in the depth of focus at the point, Z=10 µm. Meanwhile, the intensity of the O-H stretching band decreased as the focal point moved down when using the 6 µm Teflon spacer, which means the bulk water layer under the droplet is negligible. Therefore, we used 6 µm spacers because Raman spectra of the sample with 6 µm spacers better describe the structure inside the droplet, considering that the bulk water layer can be neglected within the depth of focus.

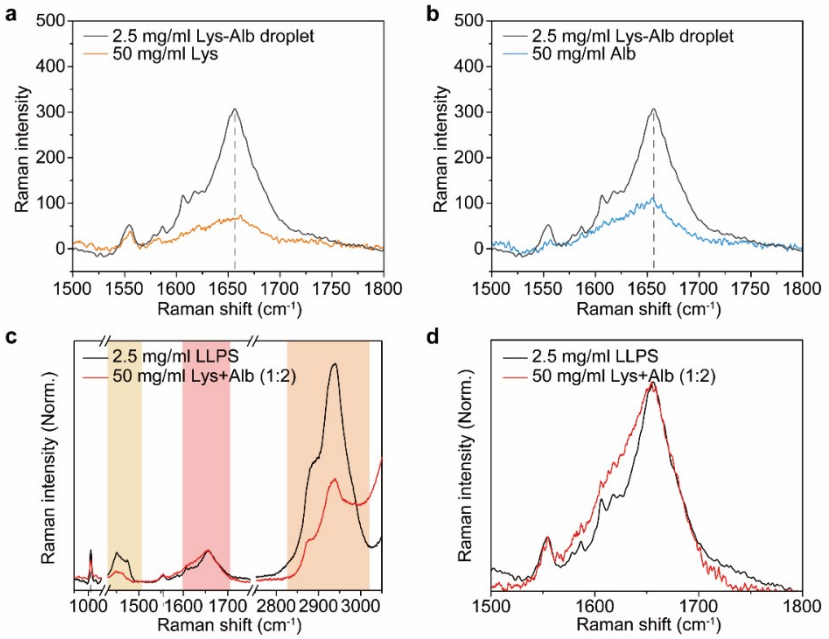

Figure S3. Raman spectra of highly concentrated Lys and Alb solution

Raman spectra in the amide I region of (a) 50 mg/ml Lys solution (orange line) and (b) 50 mg/ml Alb solution (blue line), and droplet of Lys−Alb (2.5 mg/ml each) with 10% w/v PEG (black line) for comparison. All samples were measured using 6 μm spacers. (c), (d) Normalized Raman spectra in the amide I region of the liquid droplet (black line) and protein solution (red line). The spectrum "50 mg/ml Lys+Alb (1:2)" in this figure is the sum of two Raman spectra of 50 mg/ml Lys and 50 mg/ml Alb solutions in a 1:2 ratio. To confirm the presence of Alb, Lys, and PEG inside the droplet, we considered the Raman spectra of highly concentrated Lys and Alb solutions without PEG and their combinations. The relative ratio of the two independently measured spectra (50 mg/ml Lys and 50 mg/ml Alb solutions) was adjusted to find the best combination, where the spectral lineshape of the combined spectrum agrees with the droplet spectrum. Here in this supporting information figure, we consider the case that the ratio of Lys to Alb is 1:2 (Figures S3c and S3d). In this case, the ratio of the Trp Raman signal to the amide I Raman signal shows a good agreement with that of the droplet spectrum. However, the lineshape of the amide I band of (1:2) combined spectrum differs from that of the droplet spectrum. On the other hand, as can be seen in Figures 3b and 3c in the main text, where the ratio of Lys to Alb is assumed to 1:1, we found that the lineshape of the amide I band well matches with the droplet Raman spectrum even though there is a slight difference in the intensity ratio of Trp to amide I Raman signals. In the main text (Figure 3), we showed the 1:1 combined spectrum because the lineshape of the amide I band is highly sensitive to the protein's secondary structures, and typically, the Trp Raman signal is substantially weaker than the amide I Raman signal.

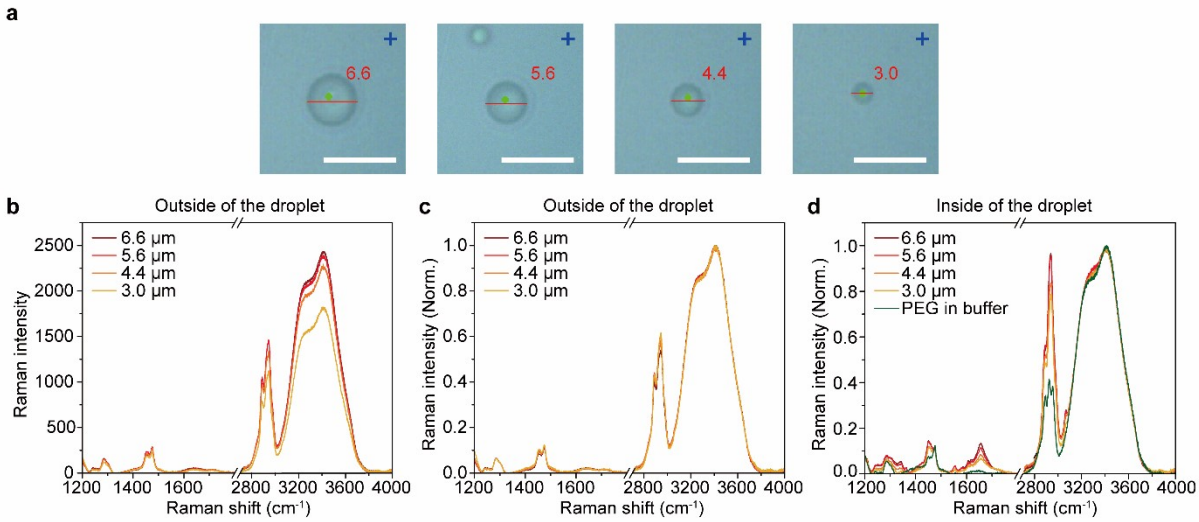

Figure S4. Normalization process to analyze droplet size-dependent Raman spectra

(a) Bright-field images of droplets with different diameters (6.6, 5.6, 4.4, and 3.0 μm). Blue spots "+" denote the x,y-positions of the focused excitation beam. Scale bar: 10 μm. The Raman spectra of the outside of each droplet change depending on the size of the droplet. This is due to the change in the z-position of the beam focus after moving the sample stage laterally along the x- and y-axis to find different droplets experimentally. (b) The background Raman spectra at the position outside of each droplet. They differ from one another due to the undesired change in the z-position of the beam focus. All these spectra are normalized so that the peak intensities of the bulk water OH bands are the same. (c) The normalized Raman spectra are shown. Not only the bulk water OH bands but also other bands, e.g., amide I bands, are nearly overlapped with one another, which indicates that this normalization procedure works well. In the main text (Figure 4), we used these normalization factors to obtain the intensity normalized Raman spectra of droplets for direct comparisons. (d) Full-range normalized Raman spectra measured for each liquid droplet (Figure 4) using the normalization factor above.

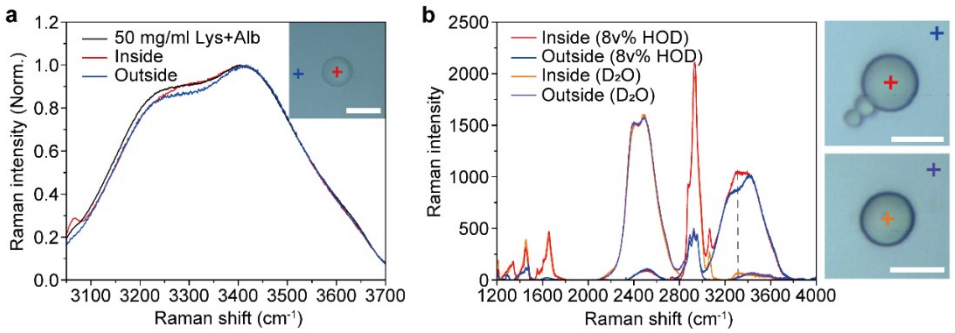

Figure S5. Spectral overlap between the N-H stretching band of protein and the O-H stretching band of water

(a) Normalized Raman spectra protein concentrated solution from the summation of 50 mg/ml Lys and 50 mg/ml Alb solution (black line) and inside/outside a single droplet of Lys−Alb with PEG (red and blue line). Inset: Bright-field images of heteroprotein droplets dissolved in Tris-buffered solution (5 mM, pH 7.4) (b) Raman spectra for inside and outside of a single droplet dissolved in an isotopically dilute buffer (red and blue line) and deuterated buffer solution (orange and purple line). Right panel: Bright-field images of heteroprotein droplets dissolved in isotopically dilute buffer (upper panel) and deuterated buffer solution (lower panel). Red and blue spots in the upper panel and orange and purple spots in the lower panel denote the position of the excitation beam inside and outside of the droplet. Scale bar: 10 µm.

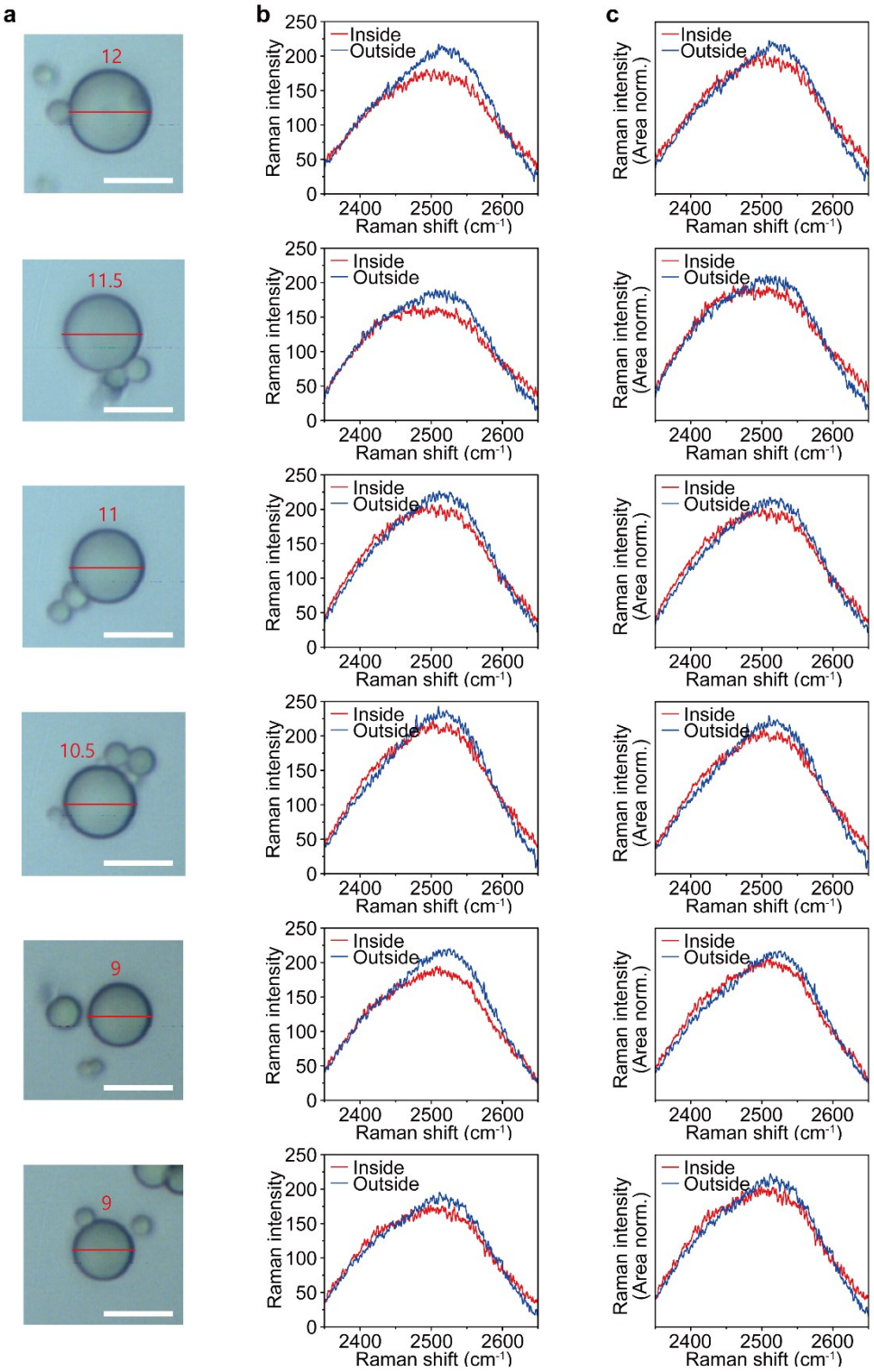

Figure S6. Reproducibility of the O-D stretching band of HOD in a single droplet

(a) Bright-field images of heteroprotein droplets with different diameters (12, 11.5, 11, 10.5, 9, 9 μm) dissolved in isotopically dilute buffer solution conducted in two different independent experiments. Scale bar: 10 μm. Raman spectra of the O-D stretching region for the inside and outside of each droplet (b) before and (c) after area-normalization. Both spectra set show consistent results, confirming the reproducibility of the quantitative and qualitative results. The fitting results (band position and fraction) of O-D stretching bands for two populations of water molecules are shown in Figures 5c and 5d.