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Supplementary Information for: Osmo-solidification of All-aqueous Emulsion with Enhanced Preservation of Protein Activity

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1. Experimental setup of the osmo-solidification process.

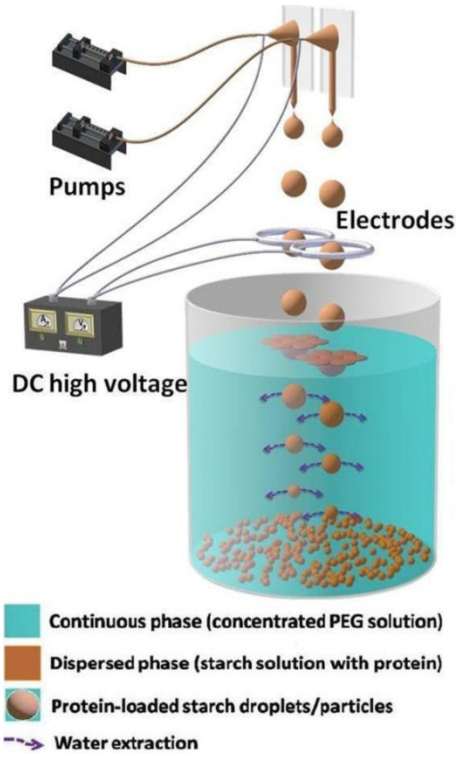

Figure S1. Schematic of the experimental setup.

To form the all-aqueous emulsion, an aqueous solution containing the matrix material, such as water dissolved with 10%*wt* starch, was used as the droplet phase, while a highly concentrated osmolyte solution, such as water dissolved with 40%*wt* PEG (Mw~8000) was used as the continuous phase. The droplet phase was pumped through soft tubing using a syringe pump (Model Lsp01-2A, Baoding Longer Precision Pump Co., Ltd.), then through a metallic needle and a tapered glass capillary, as shown in Figure S1. The flow rate of the droplet phase was 1500 ul/h. The metallic needle was connected to the direct current (DC) power supply through a wire, while the counter electrode was a metallic ring with a diameter of 4 cm positioned 1 mm beneath the capillary tip. The applied voltage between the two

electrodes was around 3.5 kV. The electro-sprayed droplets were forced to pass through the metallic ring and collected into the highly concentrated osmolyte solution. The concentrated osmolyte solution was used to extract water from the droplets, resulting in an increase in the matrix concentration, consequently triggering the droplets to solidify. The osmo-solidification approach is general and can be applied to different matrix materials, and to form particles with different sizes.

2. Osmolarities of different droplet and continuous phases of all-aqueous emulsions.

The osmolarities of different droplet and continuous phases of the all-aqueous emulsions were measured by depression of the freezing point in an osmometer (Advanced Instruments Inc, Mass, USA). The osmolarities of the matrix material solutions and concentrated aqueous osmolyte solutions are shown in following Table S1and S2, respectively.

Table S1. Table listing the osmolarities of different matrix material solutions.

Matrix material solutions			
5%*wt* starch	10%*wt* starch	15%*wt* starch	8%*wt* dextran
38 mOsm/L	77 mOsm/L	117 mOsm/L	26 mOsm/L

Table S2. Table listing the osmolarities of different concentrated aqueous osmolyte solutions.

Concentrated osmolyte solutions			
30%*wt* PEG (M_w 8000)	40%*wt* PEG (M_w 8000)	50%*wt* PEG (M_w 8000)	55%*wt* PEG (M_w 8000)
895 mOsm/L	2147 mOsm/L	4742 mOsm/L	8900 mOsm/L

3. The calculation of volumes of particles dried under high temperature and the expansion ratio of osmo-solidified particles.

Two assumptions should be taken into account when calculating the volumes of particles dried under high temperature: first, all the water in the droplet phase was extracted; second, there was no exchange between starch and PEG molecules. Based on the two assumptions, the volumes of particles dried under high temperature can be obtained by simply dividing the total mass of starch in the droplet by the theoretical density of the particle after all the water was extracted. The density of starch solution was Ds and the volume of each droplet was Vd. Thus, the total mass of each droplet (Md) can be calculated as $Md=Ds \times Vd$.

Since the concentration, by weight, of starch solution was known as w, the mass of starch inside each droplet (Ms) can be calculated as $Ms=w\times Md$. For calculating the density of the particle after complete extraction of water, a starch solution was heated up at 65°C to evaporate all the water; then the mass (Mr) and the volume (Vr) of the residues can be measured. The density (Dc) can be calculated as $Dc=Mr/Vr$.

Moreover, the volumes of particles dried under high temperature (Vc) can be calculated as Vc=Ms/Dc.

Herein we took a 10%*wt* starch solution as an example to show the calculation process. Ds was around 1μg/μl and Vd was around 1.25 μl; thus the total mass of each droplet (Md) was approximately 1.25 μg. The weight concentration of 10%*wt* starch solution was 10%, while the mass of starch inside each droplet (Ms) was 0.125 μg. The mass (Mr), volume (Vr) of the residues and calculated density (Dc) after heating that were used in calculating the theoretical density are shown in following Table S3.

Table S3. Table illustrating the parameters used in calculating theoretical density of the osmo-solidified particles.

Batch number	1	2	3
Mass of the residues (Mr) (g)	0.0961	0.095	0.0934
Volume of the residue (Vr) (ml)	0.055	0.06	0.055
Calculated Density (Dc) (μg/μl)	1.747	1.583	1.698
Average density (μg/μl)	1.676		
SD	0.0502	0.0657	0.0155

Finally, the volume of particles dried under high temperature was obtained Vc= 0.143 μl. After the volumes of particles dried under high temperature were obtained, the expansion ratio of the osmo-solidified particles was also calculated. The results of starch particles prepared by osmo-solidified 10%*wt* starch droplets in PEG (M_w~8000)

solutions at different osmolarities (895, 2147, 4742 and 8900 mOsm/L) are shown in the Table S4.

Table S4. Table illustrating the parameters used in calculating the expansion ratios of the osmo-solidified particles.

Osmolarities of PEG solution (mOsm/L)	895	2147	4742	8900
Volume of particles dried under high temperature (Vc) (µl)	0.143			
Volume of osmo-solidified particles (Va) (µl)	0.207	0.187	0.178	0.169
Expansion Ratio (Va/ Vc) (%)	144.76	130.77	124.48	118.18

4. Determination of the released proteins from osmo-solidified particles after sonication.

Lysozyme-loaded osmo-solidified particles were immersed in the aqueous dissolution media and subjected to sonication for 30s at 300W. Then a known volume of buffer solution was added and the whole mixture was centrifuged at 3000 g for 15minutes. Afterwards, the residues of the broken particles at the bottom were collected and re-dissolved in 10 ml water by stirring at 37°C for about half an hour. The resultant solution was subsequently subjected to the measurement of the amount of lysozyme by measuring the UV absorbance at 280 nm. The absorbance value was compared with those obtained in a previously measured calibration curve for lysozyme dissolved in starch aqueous solutions. In this manner, the amount of un-released lysozyme (trapped in the osmo-solidified particles) was determined.

The absorbance values of the points for the calibration curve are shown in the following Table S5.

Table S5. Table listing the absorbance values at different lysozyme concentrations for obtaining the calibration curve.

Concentration of lysozyme (μg/ml)	1	5	10	15	20
Absorbance	0.014	0.102	0.226	0.334	0.442

The regression equation was:

$$A = 0.0227c - 0.076 \quad (3)$$

where A was absorbance value, and c was the concentration of lysozyme. Correlation coefficient R=0.999, range of concentration = 1~ 20 μg/ml.

The concentration of un-released lysozyme can be calculated based on the measured absorbance values. For instance, the result was 0.38 μg/ml for osmo-solidified particles with an initial lysozyme concentration of 1.25mg/ml, therefore the amount of un-released lysozyme was 3.8 μg. The amount of total lysozyme used in the trial can be calculated as 1.25 mg/ml× 1.25 μl ×100 = 156 μg, taken into account the encapsulation efficiency as 80%, the amount of encapsulated lysozyme should be 125 μg. Therefore the amount of un-released lysozyme was only 3% of the encapsulated ones and indicating that almost all the encapsulated proteins was released out.

5. Measurement of the amount of dissolved starch in PEG phase.

A fixed volume of 10%*wt* starch solution was sprayed into five solutions of PEG (M_w 4000, 6000, 8000, 10000 and 20000) with the same osmolarity (around 2147 mOsm/L), separately. Afterwards, all osmo-solidified particles were taken out, and iodine was added into the remaining PEG solutions. The emergence of a purple color would indicate the presence of starch in the PEG solutions. To test this, an ultraviolet spectrophotometer (Nanodrop 2000c Spectrometer, Thermo Fisher Scientific Inc, USA) was used to measure the absorbance of the five PEG solutions at 560 nm. With an experimentally determined calibration curve as a function of concentration of starch solutions, the amount of starch in these five solutions can be calculated. The results and the calibration curve are shown in Figure S2.

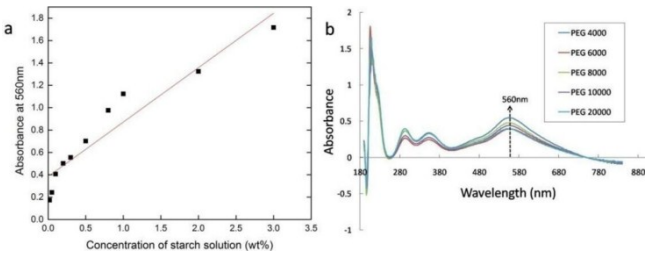

Figure S2. a) Calibration curve of different diffused starch samples. b) Absorption curves of starch in solutions of PEG with different molecular weights.

6. Porosity of osmo-solidified particles osmo-solidified by solutions of PEG with different molecular weights.

For the porosity of osmo-solidified particles by solutions of PEG with different molecular weights (M_w 4000, 6000, 8000, 10000 and 20000) at same osmolarity (around 2147 mOsm/L), particles were cut open and their cross-sections were examined using scanning electron microscopy. The porosity was subsequently measured by analyzing the SEM images using *Image J*.

7. Encapsulation efficiency of particles in osmo-solidified particles.

The encapsulated lysozyme was released from the osmo-solidified particles by sonication and dissolved in the supernant by centrifugation. Then the concentration of lysozyme in the supernant was determined by measuring the UV absorbance at 280 nm. The result was compared with the absorbance value obtained in a previously measured calibration curve for lysozyme. In this manner, the concentrations of lysozyme encapsulated in the osmo-solidified particles were determined. The encapsulation efficiency was then calculated by the following relationship:

$$EE = \frac{Measured\ amount\ of\ lysozyme\ per\ batch}{Amount\ of\ lysozyme\ initially\ added\ to\ the} \quad (4)$$

The absorbance values of the points for the calibration curve are shown in the following Table S6.

Table S6. Table about absorbance values of the points for the calibration curve.

Concentration of lysozyme (μg/ml)	1	5	10	20	30	40	50
Absorbance	0.024	0.123	0.245	0.480	0.735	0.982	1.227

The regression equation was:

$$A = 0.024c - \ 0.002 \qquad (5)$$

Where A was absorbance value; c was the concentration of lysozyme. Correlation coefficient R=0.999, range of concentration = 1~ 50 μg/ml.

8. Droplet phase dissolved with proteins surrounded by an aqueous osmolyte solution and by a conventional organic solvent.

For lysozyme, a 10%*wt* starch solution dissolved with lysozyme at 1 mg/ml was emulsified with dichloromethane (DCM) at a water-to-organic solvent ratio (1:1) using an ultrasonicator for a known period of time and power level. Unless otherwise specified, sonication was carried out for 30 seconds at 300 W. After emulsification, 10 ml of 66.6 mM phosphate buffer solution was added to the emulsion; the mixture was then centrifuged at 3000 *g* for 15 minutes to separate the dichloromethane and the buffer solution with lysozyme, where *g* referred to the gravitational constant. Residual dichloromethane was removed by placing the buffer solution under vacuum for 30

minutes. Then the protein activity assay was performed on the buffer solution. Besides, the 10%*wt* starch solution dissolved with lysozyme at 1 mg/ml was also emulsified with 10%*wt* PEG (M_w~8000) solution with similar protocols described above. After centrifugation and separation of the droplet phase from the continuous phase, the protein activity in the droplet phase was measured as a control. Same protocols were performed to measure the activity of *β*-galactosidase after emulsified with dichloromethane (DCM) and 10%*wt* PEG (M_w~8000) solution separately. The activity of α-amylase dissolved in an 8%*wt* dextran solution was also measured using the same protocol after emulsification in dimethyl sulphoxide (DMSO) and 10%*wt* PEG (M_w~8000) solution separately. Each experimental group was conducted in triplicate.

9. Determination of residual water content in the osmo-solidified particles.

Herein we took a 10%*wt* starch solution as an example to describe how the residual water content in the osmo-solidified particles was determined. Starch particles osmo-solidified by 40%*wt* PEG (M_w~8000) solution were collected and weighed; the measured mass is shown in Table S7. Then these particles were placed inside an oven at 85°C and incubated for overnight to fully evaporate the water. Afterwards, these particles were weighed again and the mass after heating is also shown in Table S7.

Assuming that only water, but not the matrix material or the proteins, can be evaporated, the amount of residual water content can be calculated according to the following equation:

$$Residual\ water\ content = \frac{Initial\ mass - \ Mass\ after\ heating}{Initial\ mass} \times 100\% \qquad (6)$$

Table S7. Table illustrating the experimental determination of residual water content of the osmo-solidified particles.

Batch number	1	2	3
Initial mass (mg)	11.3	11.7	10.9
Mass after heating (mg)	10.1	10.5	9.8
Residual water content (%)	10.6	10.3	10.1
Average residual water content (%)	10.3		

10. Lysozyme activity assay.

Lysozyme is capable of digesting bacterial cell walls and *Micrococcus lysodeikticus* cells were used as the substrate in the activity assay. The rate of lyses of *Micrococcus lysodeikticus* cells by lysozyme was used to estimate the biological activity of the protein and the turbidity of the cell suspension was measured at 530 nm by an ultraviolet spectrophotometer. Briefly, a volume of 2.0 ml of *Micrococcus lysodeikticus* cells in suspension was mixed with 0.2 ml lysozyme solution. The decrease in absorbance was monitored during a total period of 2 minutes. Then the transparence value was calculated by Beer–Lambert law. The activity of test sample

was calculated by comparing the change of transparence values of test sample with standard lysozyme according to the equation:

$$Activity\ value = \frac{T_{t2} - T_{t0}}{T_{s2} - T_{s0}} \times 200U/ml \times dilutedtimes \quad (7)$$

Ts0 was the transparence value of standard lysozyme at the beginning of the absorbance measurement. Ts2 was the transparence value of standard lysozyme after 2 minutes. Tt represented the transparence value of the test sample.

The unit of lysozyme activity was expressed as U/mg. 1 U indicated the amount of lysozyme that catalyzed the reaction of 1 μmol/ml of substrate per minute. The lysozyme activity was the number of U per ml divided by the concentration of protein in units of mg/ml.

The activity value of standard sample of lysozyme was 80000 U/mg. The standard sample was diluted before measuring its absorbance to achieve an activity value of 200 U/ml. The samples were diluted by 1250 folds in our trials.

The relative activity of each test group can be calculated by Equation (2) after all the activity values were obtained by the UV spectrophotometer.

11. β-galactosidase activity assay.

The activity of *β*-galactosidase was assayed by measuring the amount of ortho-nitrophenol (ONP) released from o-nitrophenol-*β*-galactopyranoside (ONPG) due to the presence of *β*-galactosidase. Briefly, the protein solution (0.1 ml) was mixed with 3.4 ml of 10 mM ONPG in a 0.1 M phosphate buffer at pH 7.0, and held at 30°C for 10 minutes. The reaction was stopped by adding 1.5 ml of 0.5 mM Na_2CO_3 and the absorbance of ONP was measured at 420 nm. The unit of *β*-galactosidase activity was expressed as U/mg. 1 U was defined as the amount of *β*-galactosidase that hydrolyzed ONPG to 1 μmol ONP per minute of the reaction time. Each experimental group was conducted in triplicate. The activity values E were quoted as U/mg.

Different known amounts of ONP were dissolved in 0.1 M phosphate buffer at pH 7.0. The absorbance of the solutions at 420 nm was measured using a UV-vis spectrophotometer. The resultant calibration curve of absorbance as a function of the ONP concentration was used to calculate the concentration of ONP. (Figure S3)

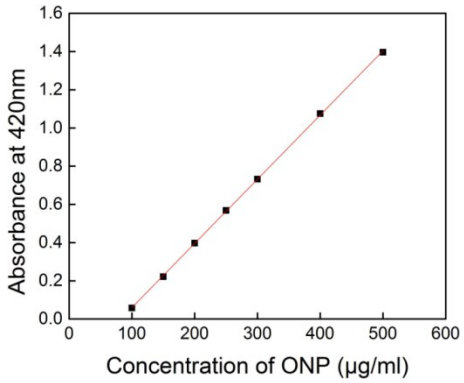

Figure S3. Calibration curve of different ONP samples.

The regression equation was

$$A = 0.003c - \ 0.227 \quad (8)$$

Where A was absorbance value; c was the concentration of ONP. Correlation coefficient R=0.999, range of concentration = 100~500 μg/ml. Then the activity value of *β*-galactosidase can be calculated by the following equation:

$$E = 3.06 \times A \times diluted\ percentage\ /T \quad (9)$$

Where E was the activity value of *β*-galactosidase; A was the absorbance value; 3.06. When the absorbance value was 1, 3.06 μmol/ml ONP was produced; T was the time of the reaction between *β*-galactosidase and ONPG. T was about 10 minute in our experiment. The *β*-galactosidase as received was diluted by 1000 times in our trials.

The relative activity of each test group was calculated by Equation (2) after all the activity values were obtained as described.

12. α-amylase activity assay.

α-amylase is an enzyme that catalyzes the hydrolysis of starch. The level of α-amylase in various fluids of the human body is of clinical significance in the diagnosis of disease states including pancreatitis and diabetes. There are plenty of methods for measuring the activity of α-amylase. Herein we used the commercial EnzChek®Ultra Amylase Assay Kit to measure its activity. This kit contained a starch derivative, the DQTM starch, which was labeled with a fluorescent dye to such a degree that the

fluorescence was quenched. This substrate was efficiently degraded by amylase; the degration leaded to a reduction of the quenching and yielded highly fluorescent fragments. The accompanying increase in fluorescence was proportional to amylase activity and can be monitored with a fluorescence microplate reader, using standard fluorescein filters. The experimental procedures were based on the standard protocols suggested by the EnzChek®Ultra Amylase Assay Kit. The concentration of α-amylase was defined as U/ml, where one unit was defined as the amount of enzyme required to release 1 mg of maltose from starch in 3 minutes at 20°C and pH 6.9. Each experimental group was conducted in triplicate.

To obtain a calibration curve, different known amounts of α-amylase were first dissolved in buffer solution. Then the DQ starch was dissolved to reach a working concentration of 200 μg/ml. The resultant α-amylase solution and DQ starch mixtures were pipetted in volumes of 50 μl separately into the wells of a 96-well plate, which was then examined in a fluorescence microplate reader at an excitation wavelength of 490 nm. The results have been summarized in the form of a calibration curve shown in Figure S4.

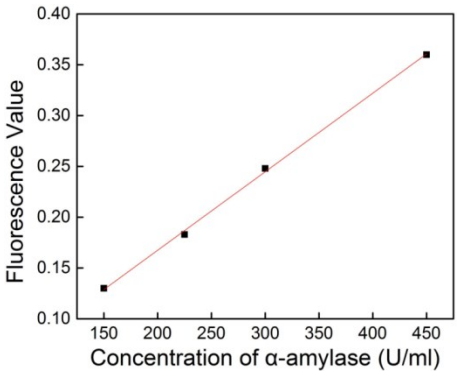

Figure S4. Calibration curve of concentrations of different α-amylase samples.

The regression equation was

$$a = 0.0008c + \ 0.013 \qquad (10)$$

Where A was absorbance value; c was the concentration of α-amylase. Correlation coefficient R=0.999, range of concentration = 150~450 U/ml.

Using the calibration curve, the activity of α-amylase following freeze-drying and osmo-solidification were measured. The protocols used to release the encapsulated α-amylase were the same described before. The activity of the protein in the supernatant was measured using the EnzChek®Ultra Amylase Assay Kit. A 250 U/ml pure α-amylase solution was used as a control.

The relative activity of each test group can be calculated by Equation (2).