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All-Aqueous Electrosprayed Emulsion for Templated Fabrication of Cytocompatible Microcapsules

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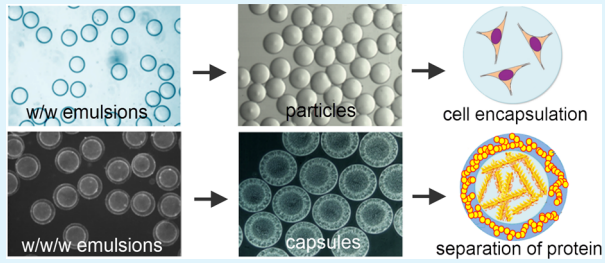
Supporting Information

ABSTRACT: Encapsulation of biomolecules and cells in hydrogel capsules via emulsion templating frequently induces an irreversible loss of bioactivity, because of the use of nonaqueous solvents. Here, we introduce an all-aqueous electrospray (AAE) approach to generate aqueous two-phase emulsion droplets, and we use them as templates to fabricate microcapsules with preserved cell viability. The approach allows formation of monodisperse microparticles with tunable sizes, variable compositions, and interior architectures in a mild gelation process. This technique potentially benefits a variety of new biomedical applications, such as delivery of bioactive proteins, transplantation of living cells, and assembly of cell-mimicking structures.



KEYWORDS: *all-aqueous droplets, w/w/w double emulsion, core−shell microcapsules, biomolecular assembly, electrospray, cell encapsulation*

1. INTRODUCTION

Design of the architecture of microparticles and microcapsules is frequently demanded in diverse biological studies and biomedical applications, such as drug delivery,[1,2] diagnostic imaging,[3,4] and three-dimensional (3D) culture of cells.[5,6] In these applications, emulsion droplets preloaded with gel precursors are often used as liquid templates for preparation of hydrogels. By delicate control over the sizes and structures of emulsion droplets, the properties and functionality of microparticles and capsules can be tailored.[7−9] Despite the tremendous success of this approach in various applications, the frequent use of organic solvents as emulsion-forming liquids often raises concerns on the bioactivity of the encapsulated species. For instance, proteins and lipids encapsulated in the water-in-oil (w/o) emulsion are subjected to unexpected conformational changes[10] and oxidation[11] after exposing to the water/oil interfaces. Moreover, residual organic solvents left in the gel can also induce cytotoxicity. In avoiding the use of organic solvents, the aqueous two-phase system has been shown to be a feasible alternative.[12,13]

Water-in-water (w/w) emulsions are formed when two incompatible solutes, such as dextran and polyethylene glycerol (PEG), are added to water above the threshold concentrations of phase separation. As biocompatible formulations, w/w emulsions can better preserve the activity of biomolecules and viability of cells than their counterparts involving both water and oil. As an example, the aqueous two-phase system has been widely used as biocompatible media for separation of biomolecules[14,15] and cells.[16−18] Bioengineered extracellular matrixes[19] and porous scaffolds[20] made from aqueous two-phase droplets exhibit enhanced permeability to nutrients and excellent cytocompatibility. These evidence suggest that the use of all-aqueous emulsion droplets creates mild conditions for hydrogel gelation,[21] which is better suited for encapsulation and delivery of bioactive species, such as cells, proteins, lipids, and DNA.

While all-aqueous emulsions represent a class of biocompatible templates for hydrogel formation,[17−19] the lack of control over the dimensions and structures of the produced w/w emulsions challenges the wider use of this approach. Traditional techniques of emulsification, such as ultrasonic agitation[22] and homogenization,[23] often yield droplets with a broad size distribution and diverse emulsion structures. Microparticles obtained by solidifying these emulsions yield unpredictable sizes, depending on the specific formulations of the emulsion mixture and the conditions of mixing. The microfluidic approach that allows the delicate manipulation on the w/o emulsions is not easily applicable for generation of w/w emulsions. The underlying challenge is that the ultralow interfacial tension[24] between two immiscible aqueous phases delays the breakup of the dispersed jet phase[25] and often results in droplets with polydisperse sizes. To control the diameters of the w/w emulsions, perturbation has been applied to enforce the breakup of the w/w jet in the microfluidic devices.[26−28]

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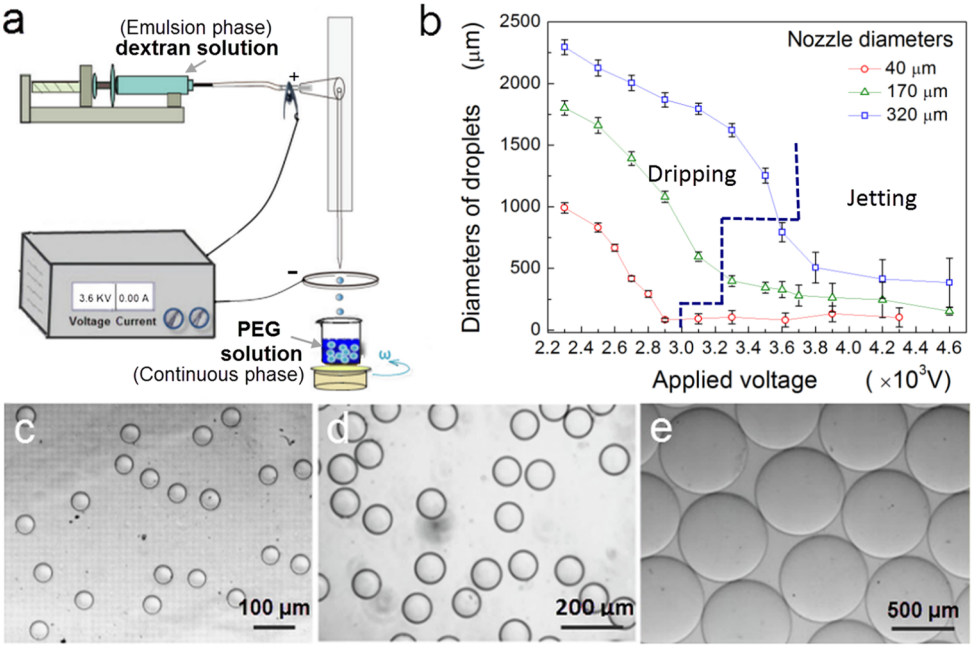

Figure 1. Generation of water-in-water (w/w) emulsions with tunable sizes via all-aqueous electrospray: (a) schematic of the experimental setup. (b) A plot showing that the diameters of the w/w emulsions (D) decrease as the applied voltage increases or the diameter of the glass nozzle reduces. (In panel b, the symbols represent the diameters of droplets produced by varying the diameter of the glass nozzles at (○) 40 μm, (△) 170 μm, and (□) 320 μm, respectively.) (c–e) Optical microscope images of the monodisperse w/w emulsions with tunable sizes (the nozzle diameter is 40 μm and the flow rate of the emulsion phase is 0.5 mL/h): (c) the applied voltage is U = 2.92 kV, the rotation speed of the collection bath is ω = 1 rad/s, and the polydispersity of droplets is P = 4.6%; (d) U = 2.85 kV, ω = 0.8 rad/s, and P = 3.1%; and (e) U = 2.67 kV, ω = 0.5 rad/s, and P = 2.5%.

While the approach can efficiently break up many nonviscous w/w jets, it remains challenging to break up a viscous w/w jet into uniform droplets.[29] Such challenge limits the use of droplets involving viscous aqueous two-phase systems, such as protein-rich and polysaccharide-rich phases in biomedical applications. Therefore, a general approach to form all-aqueous emulsions from viscous solutions is needed.

Here, we combine the technique of electrospray[29−31] and an emulsion-templating approach[32−34] to generate hydrogel microparticles and capsules. The approach allows the production of cytocompatible hydrogel particles and capsules with variable compositions, highly tunable sizes, and designer architectures. The robustness and versatility of this approach would benefit potential industrial and biomedical applications, such as the protein delivery, cell encapsulation, and cell-mimicking studies.

2. EXPERIMENTAL SECTION

2.1. All-Aqueous Electrosprayed Formation of Droplets. To form dextran-in-PEG single emulsion droplets, the droplet phase is a 15 wt % dextran (M_w = 500 000, Shanghai Ryon Biological Technology Co., Ltd.) solution and the continuous phase is 8 wt % polyethylene glycol (PEG, M_w = 20 000, Sigma) solution. The emulsion phase is pumped through soft tubing, a metallic needle and a tapered glass capillary in sequence, as shown in Figure 1a. The metallic needle is connected to the cathode of a direct current (DC) power supply through a wire, so that the emulsion phase is positively charged. A metallic ring with an enclosed diameter of 5 cm is positioned 1 mm beneath the capillary tip, which is used as the counter electrode. The applied voltage between the two electrodes ranges from 2.2 kV to 4.6 kV. The flow rate of the emulsion phase is 0.5 mL/h. The electrosprayed droplets are forced to pass through the metallic ring and collected in a rotating bath (0.5−1 rad/s) containing the PEG-rich continuous phase. A solution of 0.1 wt % fibrils of lysozyme (Shanghai Ryon Biological Technology Co., Ltd.) is added to both the emulsion and continuous phases to stabilize the collected emulsion droplets. The lysozyme fibrils are prepared by heating 1 wt % lysozyme solution at 65 °C for 60 h.[35]

The water/water/water (w/w/w) double emulsions are generated by dispensing a single-phase solution containing 5 wt % dextran and 1 wt % PEG (M_w= 20 000) into a continuous phase of the 8 wt % PEG (M_w = 8000) solution.[36] The experimental setup and procedures are the same as described for the generation of w/w single emulsions. The applied voltages are adjusted from 2.6 kV to 2.85 kV for making droplets with different diameters. The emulsion subsequently phase-separates into two immiscible phases, which make up the PEG-rich core and the dextran-rich shell of the double emulsion.

2.2. Fabrication of Particles and Capsules. *Polyethylene Glycol Diacrylate (PEGDA) Microparticles.* A mixture of solutions dissolved with 30 wt % tripotassium phosphate (K_3PO_4) and 10 wt % PEGDA (M_w = 700, Sigma) respectively is centrifuged at 6000 rpm for 5 min, yielding a PEGDA-rich top phase and a K_3PO_4-rich bottom phase. The PEGDA-rich and K_3PO_4-rich phases are separately extracted as the emulsion and continuous phases to form droplets through all-aqueous electrospray. Irgacure 2959 (0.05 wt %, Sigma) is added into the continuous phase as the photoinitiator. The obtained PEGDA-rich emulsion droplets are then solidified upon exposure to UV for 30 s using a UV light source (Spot Cure, USHIO).

Collagen Particles. A solution with 1.5 mg/mL collagen (type I, rate tail, Life Technology Co., Ltd.) and 10 wt % dextran is used as the emulsion phase. This emulsion phase is

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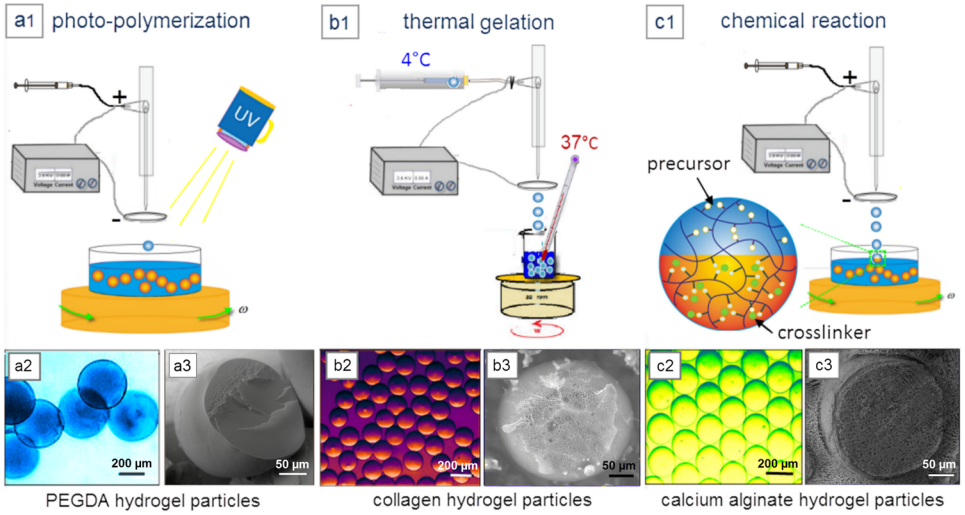

Figure 2. Schematics and microscope images of hydrogel particles fabricated by solidifying water-in-water emulsions via different strategies: (a1−a3) PEGDA particles are formed when PEGDA-in-K_3PO_4 emulsion droplets are exposed to the UV light; (b1−b3) collagen microspheres with highly porous structures can be prepared by thermal gelation of 1.5 mg/mL collagen precursors in the dextran-in-PEG emulsion droplets; and (c1−c3) calcium alginate particles are obtained by chemical cross-linking of 1.5 wt % sodium alginate in the templates of 10 wt % dextran-in-8 wt % PEG emulsions. The optical microscope images of the hydrogel particles are shown in panels a2, b2, and c2 (scale bar = 200 μm). Particles are displayed with different color schemes to enhance the contrasts. The size polydispersity of PEGDA, collagen, and alginate particles are 6.8%, 4.8% and 3.2% respectively. The corresponding scanning electron microscope (SEM) images are shown in panels a3, b3, and c3 (scale bar = 50 μm).

kept at 4 °C before injection into the 8 wt % PEG continuous phase via electrospray. The collected emulsion droplets are heated at 37 °C for 12 h to form collagen particles.

Calcium Alginate Particles. To fabricate alginate particles, the emulsion phase is dissolved with 1.5 wt % sodium alginate (brown algae, Sigma) and dextran with different concentrations (5, 10, and 15 wt %). The emulsion droplets are generated by electrospray and solidified in the continuous phase of 8 wt % PEG and 1.5 wt % $CaCl_2$ (Sigma).

Collagen Capsules. Collagen capsules are prepared by using w/w/w double emulsion as templates. The emulsion phase is composed of 1.2 mg/mL collagen, 5 wt % dextran, and 1 wt % PEG, while the continuous phase is an 8 wt % PEG solution suspended with 0.1 wt % lysozyme fibrils. The emulsion phase is preserved at 4 °C before dispensing into the continuous phase at 25 °C. After the formation of double emulsions, the collagen in the outer shell phase of the double emulsion is cross-linked by adding 0.2 wt % sodium dextran sulfate (M_w = 40 000) to the continuous phase.

2.3. Optical and Scanning Electron Microscopy. Optical images of the all-aqueous emulsions are taken using a high-speed camera (Phantom v9.1) connected to an optical microscope (Model AE2000, Motic, Inc.). The morphology of hydrogel particles is observed using the cryo-SEM (Hitachi, Model S3400). Hydrogel samples are washed with water and frozen on the cooling stage at −20 °C. In the vacuum chamber of the electron microscope, the ice is slowly evaporated from the sample surface. The microstructures of hydrogel are captured immediately after the removal of the superficial ice.

2.4. Encapsulation of Amylase Protein in Alginate Particles and Protein Activity Test. A quantity of 1 wt % β-amylase (Shanghai Ryon Biological Technology Co., Ltd.) is dissolved into the emulsion phase of water with 10 wt % dextran and 1.5 wt % sodium alginate. This emulsion phase is electrosprayed into a collecting solution of water with 1.5 wt % $CaCl_2$ and 8 wt % PEG, forming calcium alginate particles. To measure the amount of amylase encapsulated in the particles, the particles (1 mL) are transferred into 2 mL phosphate buffered saline (PBS) solution. The standard release curve of amylase is tested at 37 °C and the PBS medium is changed every half hour. After 6 h, particles are dissolved in 5 wt % sodium citrate (Sigma) solution to fully release the remaining amylase in particles. The concentration of the released amylase is measured based on the absorbance peak at 269 nm using a spectrophotometer (NanoDrop 2000c, Thermo Scientific). The efficiency of the encapsulation of amylase is calculated by dividing the total amount of amylase released from the particles by the total amount of amylase dissolved in the emulsion phase. A dextran-free emulsion phase of water with 1 wt % amylase and 1.5 wt % alginate is used for comparison.

The activity of the amylase released from the particles is tested by digesting 1 wt % starch (Aladdin Chemistry Co., Ltd.) solution for 1 h. The amount of undigested starch is determined by mixing the samples with a 10 wt % povidone−iodine solution (Banitore) at the volume ratio of 10:1, followed by measuring the UV-vis absorbance at 570 nm. The digested starch is calculated from the total amount of starch minus the undigested starch. A fresh amylase solution with the same concentration is used as a control to digest starch under the same conditions. The relative activity of amylase released from the particles is expressed as the ratio of the digested starch in the particle group to that in the control group.

2.5. Cell Immobilization and Viability Test. The human embryonic kidney 293 cell line (HEK 293) is transformed from a culture of normal human embryonic kidney cells. The cells are cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with 10 wt % fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) as supplements. To encapsulate cells into hydrogel particles, ~10^5 cells are suspended into a mixture of 2 wt % alginate and 10 wt % dextran as the emulsion phase. After preparation of the alginate particles in 1.5 wt % $CaCl_2$/10 wt % Pluronic F-68 (Sigma−Aldrich) solution, the obtained particles are transferred

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into DMEM and then labeled with the commercial Live/Dead assay kit, L3224 (1 μL ml^{-1}, Life Technologies). After incubation at 37 °C for 45 min, the particles are washed twice by DMEM and observed using fluorescence microscopy. The living and dead cells are distinguished by the green and red fluorescence, and their numbers are statistically measured by counting at least 20 particles. To test the cytocompatibility of the alginate particles, we incubate the hydrogel particles (with and without dextran) in the culture medium for 1 and 3 days. The extracted culture medium then is subsequently used for cell culture with a seeding density of 5×10^4 cells in each well. Fresh culture medium is used as the control. The viability of the HEK 293 cells are then evaluated using the CellTiter96 AQueous nonradioactive cell proliferation (MTS) assay (Promega, USA). The values of the cell viability are expressed as the ratio of the living cells in the particle groups, relative to that in the control group. For each sample group, the resultant cell viability values are a mean of the values obtained in at least three separate trials. The normalized cell viabilities in the pure alginate and alginate/dextran particles are compared using unpaired *t*-tests. A *P*-value <0.05 is considered to be statistically significant.

2.6. Detection of Amyloid Fibrils in Core−Shell Microcapsules. A mixture of 1.2 mg/mL collagen and 0−1.5 mg/mL lysozyme fibrils are suspended into an emulsion phase dissolved with 5 wt % dextran and 1 wt % PEG. The lysozyme fibrils in the emulsion phase are labeled with 5 μM thioflavin T (Shanghai Ryon Biological Technology Co., Ltd.). The emulsion phase is dispersed into an 8 wt % PEG solution to form core−shell structured droplets. Distribution of lysozyme fibrils (stained with ThT) in the double emulsion droplet is visualized under the fluorescence microscope (DMIL LED Fluo, Leica). The intensity of the fluorescence in the core, the shell and the background is measured respectively by image analysis using the open-source software, ImageJ. The relative fluorescence intensity of the core (or the shell) phase is expressed as the ratio of the fluorescence intensity in the core (or shell) phase to that in the surrounding PEG-rich continuous phase.

3. RESULTS AND DISCUSSION

3.1. Electrospray Generation of Water-in-Water (w/w) Emulsions. Monodisperse w/w emulsions with tunable sizes are generated with an all-aqueous electrospray (AAE) approach, as illustrated schematically in Figure 1a. As a model system of aqueous two-phase emulsions, a dextran-rich phase is charged positively and breaks up into droplets at the end of a capillary nozzle. These charged droplets are forced to pass through the center of a metallic ring, which is charged negatively and positioned 1 mm beneath the capillary nozzle. This setup ensures that all droplets can pass through the metal ring before collection in the continuous phase of the PEG solution. The electrostatic force (F_e) helps to pull droplets toward the counter electrode, while the water/air surface tension holds the droplets at the nozzle of the glass capillary. In this dripping regime, the charged droplets will pinch off the capillary nozzle when the gravitational and electrical forces balance with the surface tension force,[37] which can be expressed as

$$\rho V g + F_e = \pi d \sigma \quad (1)$$

where the symbols ρ and V denote the density and volume of the droplet phase, respectively; g is the gravity constant; d is the diameter of the capillary nozzle; and σ is the surface tension.

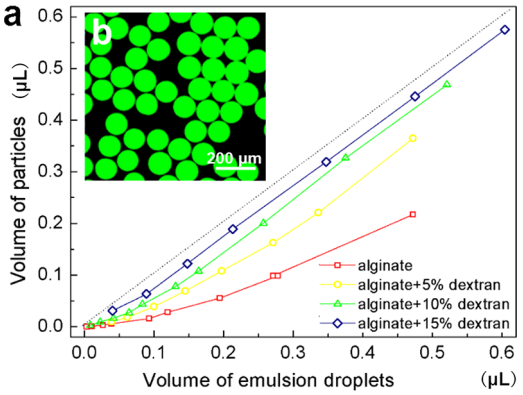

Figure 3. (a) Volume of alginate particles scales with the volume of the emulsion templates. With the increasing concentration of dextran in the droplet phase, the water inside each droplet is increasingly retained, so the volume of the obtained particles increases. (b) A fluorescence microscope image showing that dextran (M_w = 500 000) molecules are physically trapped in the alginate particles. Dextran is tagged with fluorescein isothiocyanate. The concentration of sodium alginate in the emulsion phase is 1.5 wt %.

Table 1. Comparison Showing the Enhancement in the Loading Efficiency of β-Amylase in Calcium Alginate Particles Formed with Templates of Dextran-in-PEG Droplets[a]

	encapsulation efficiency of amylase	amylase activity
alginate particles	30% ± 2%	96% ± 2%
alginate−dextran particles	47% ± 3%	94% ± 4%

[a]Amylase released from the two types of particles demonstrates equally excellent enzyme activity in digesting starch. A freshly prepared amylase solution is used as a control in measuring the enzyme activity.

With the same nozzle diameter d and surface tension σ, an increase in the electrostatic force should decrease the droplet volume V. Experimentally, the diameters of droplets (D) decrease sharply as the applied voltage (U) increases, as shown in Figure 1b. When the applied electrical force increases further, dripping of the droplets is no longer observed and the equality assumed in eq 1 is no longer satisfied:

$$\frac{F_e}{\pi d \sigma} > 1 - \frac{\rho V g}{\pi d \sigma}$$

Instead, the electrical force overcomes the surface tension of the emulsion droplets, yielding an electrical jetting regime (see Figures S1 and S2 in the Supporting Information). In this jetting regime, the collected droplets are polydisperse in size due to the formation of satellite droplets. Besides increasing the applied voltage, the size of the droplets can also be reduced by using capillary nozzles with a smaller diameter, d, as confirmed in Figure 1b. Consequently, monodisperse w/w emulsions with diameters ranging from 45 μm to 2 mm can be easily modulated by varying the nozzle diameter and the applied voltage (see Figures 1c−e). The polydispersity of the resultant w/w droplets varies from 3.6% to 6.7%, which is almost comparable to that generated by the microfluidic approach.[26,36] It should be emphasized that the proposed all-aqueous electrospray is also applicable to generate w/w emulsions using other aqueous two-phase systems, such as PEGDA-in-K_3PO_4 emulsions (see Figure S3 in the Supporting Information).

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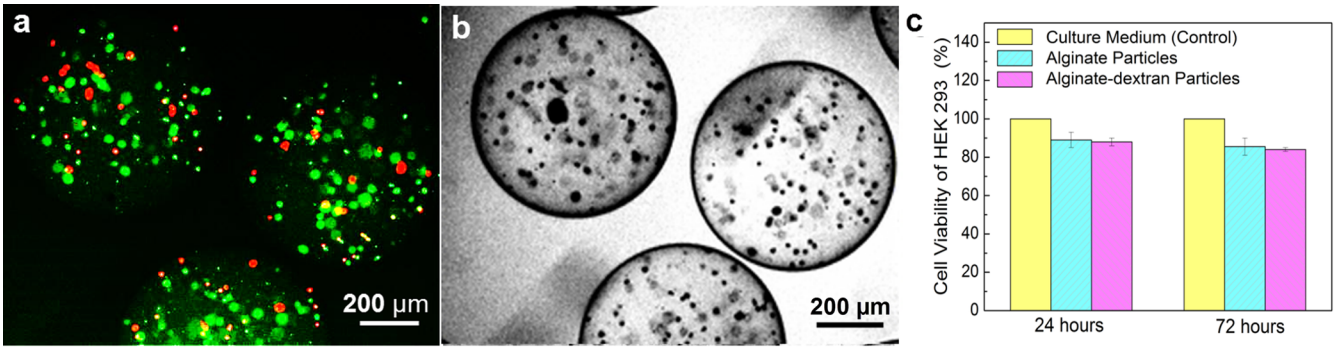

Figure 4. Human kidney cells (HEK 293) are encapsulated in the alginate particles fabricated through the AAE approach. (a) Fluorescence and (b) bright-field microscope images of alginate-dextran particles encapsulating HEK 293 cells. The living and dead cells are stained with the green and red fluorescence colors, respectively. (c) The use of w/w emulsions as templates in the fabrication of alginate particles does not induce cytotoxicity, as demonstrated in the cell viability test of the human kidney cells HEK293 after *in vitro* cell culture for 24 and 72 h.

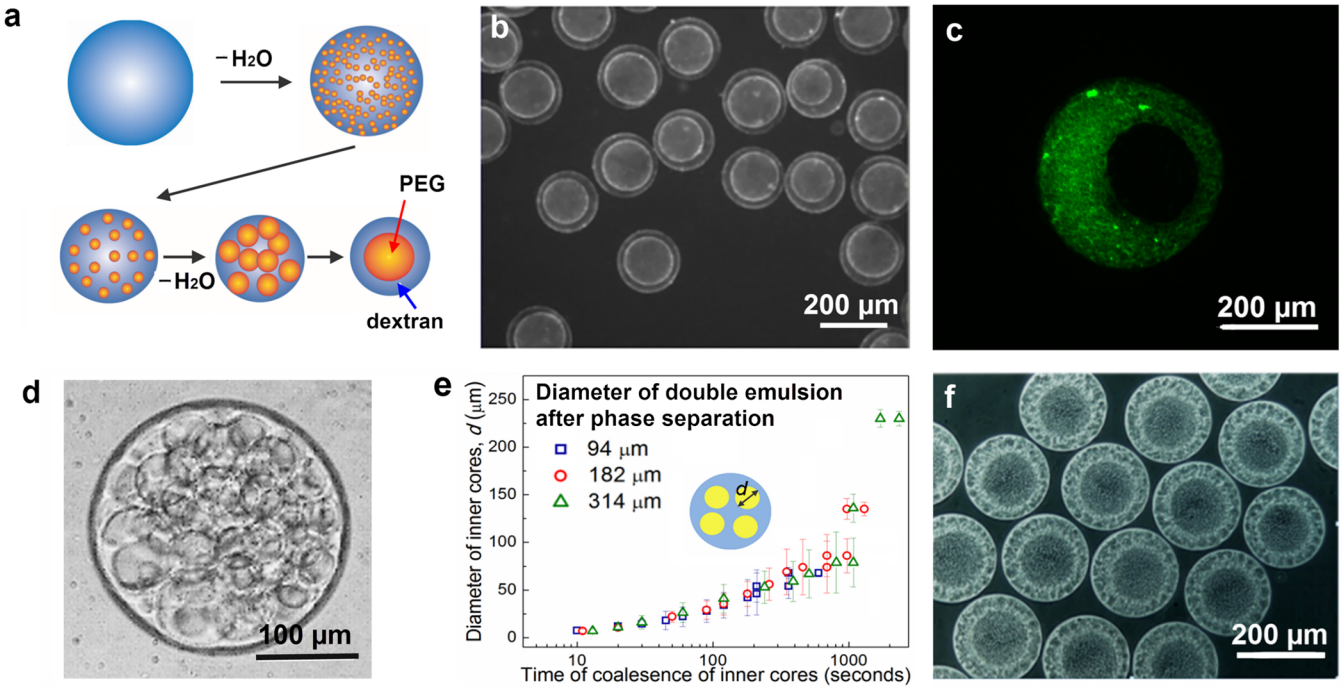

Figure 5. Fabrication of core−shell structured collagen capsules by using w/w/w double emulsions as templates. (a) Schematic illustration of the transition of emulsion structures from w/w single emulsion to w/w/w double emulsion. (b) Phase-contrast microscopy image of w/w/w double emulsion droplets fabricated through the AAE approach. (c) Collagen selectively partitions into the dextran-rich shell phase (pH 6), as confirmed by the green fluorescence of FITC-collagen emitting from the PEG-in-dextran-in-PEG double emulsion templated collagen capsules. (d) Optical microscopy image of the collagen capsule containing multiple inner compartments of the PEG-rich droplets. (e) Evolution of the capsule structure is kinetically controlled by the growth and coalescence of PEG-rich droplets, as shown by the measured average diameter of the PEG-rich cores, as a function of the phase separation time. (f) Phase-contrast microscopy image of collagen capsules after phase separation for 25 min.

When compared with the microfluidic approach, the AAE enables efficient generation of monodisperse w/w droplets with a broader range of viscosity. During the formation of aqueous droplets from the two approaches, the balance between the viscous effect and surface tension effect can be evaluated by the Ohnesorge number,[38] *Oh*, which is defined by

$$Oh = \frac{\eta}{\sqrt{\rho\sigma D}} \tag{2}$$

where η and ρ are the dynamic viscosity and density of the droplet phase, and σ is the interfacial tension between the droplet and the surrounding phases. For the microfluidic dispensing of w/w emulsions from a viscous emulsion phase, the viscous force dominates over the interfacial tension and inertia forces. This is exacerbated by the ultralow value of interfacial tension of two immiscible aqueous phases[24] (10^{-4}–10^{-1} mN/m), yielding a large *Oh* number of 10–10^2 (see Figure S4 in the Supporting Information). Consequently, a viscous aqueous phase typically forms a long and stable jet in the immiscible continuous phase. When the same fluid is dispersed through air, the surface tension of the dispersed phase increases to ∼70 mN/m; correspondingly, the *Oh* number decreases to 10^{-1}–10^0. Specifically, to form monodisperse dextran droplets shown in Figure 1, the *Oh* number varies from 0.1 to 0.4 in the all-aqueous electrospray, suggesting the interfacial tension plays a dominating role in the breakup of the jet. In comparison, the *Oh* number varies from 5 to 20 in the approach where a w/w jet is perturbed in the microfluidic

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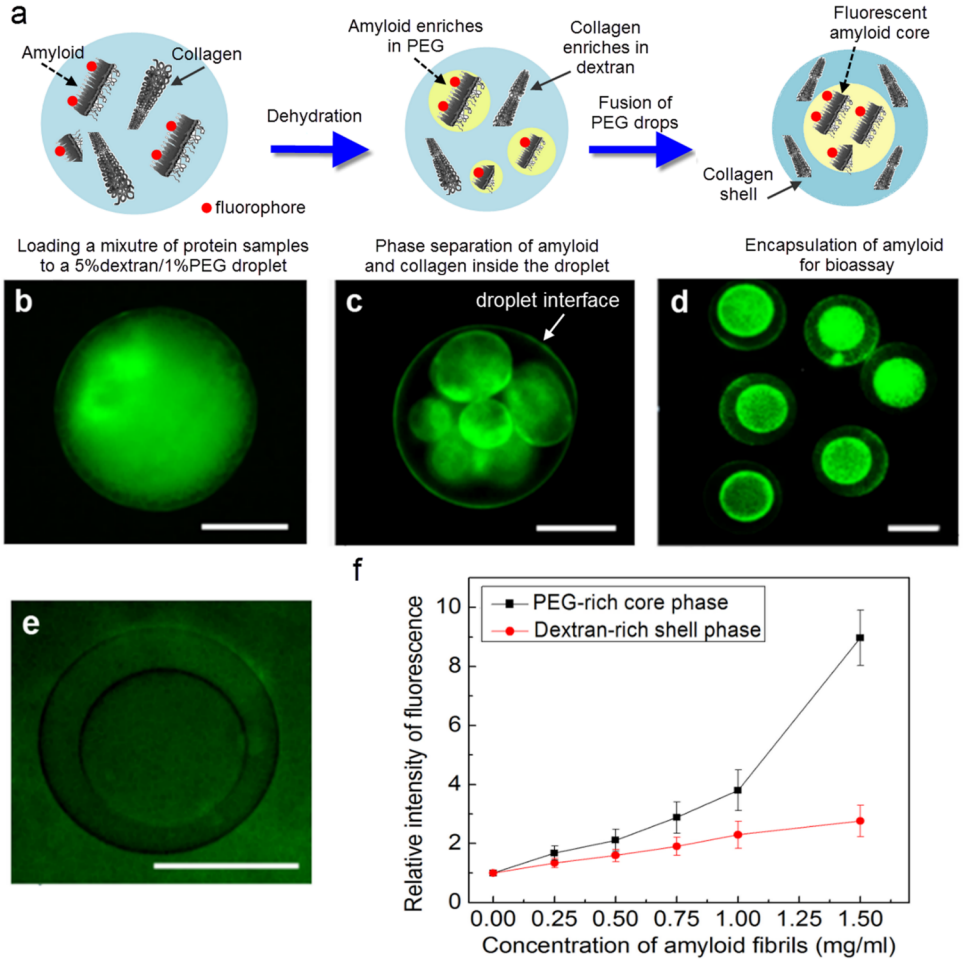

Figure 6. Detection of amyloid proteins by using w/w/w double emulsion templated microcapsules. (a) Schematic illustrating the separation of diseased amyloid fibrils from collagen fibers in a phase-separated emulsion droplet. (b–d) The 0.15 wt % lysozyme fibrils stained with 5 μM thioflavin T (ThT) are selectively loaded inside the collagen capsules. The ThT-dyed fibrils emit green fluorescence upon excited by a blue-colored spectrum. (e) In comparison, unbounded ThT molecules do not accumulate in any liquid phases and are quickly released to the continuous phase. Scale bar is 200 μm. (f) As the concentration of amyloid fibril increases, the intensity of fluorescence in both the PEG-rich core and the dextran-rich shell increases, relative to that of the background.

channels. The dramatic effect of the enhanced interfacial tension on the dispensing of viscous fluids is further confirmed by the direct formation of uniform droplets from a viscous solution with 15 wt % dextran and 1.5 wt % sodium alginate ($\eta \approx 4 \times 10^2$ mPa s) at the nozzle tip in the AAE approach. The wider applicability of the AAE approach to generate w/w emulsions will create new opportunities to fabricate hydrogel spheres for encapsulation of biomolecules, drugs, and cells.

3.2. Fabrication of Monodisperse Hydrogel Particles, Using w/w Emulsions as Templates. Hydrogel particles with a polydispersity of <5% can be fabricated using the templates of monodisperse water-in-water emulsions. Here, we demonstrate the versatility and robustness of this approach by fabricating hydrogel samples belonging to three different common schemes of gelation, as illustrated in the schematic shown in Figure 2. Solidification of w/w emulsions can be triggered optically (Figures 2a), thermally (Figures 2b), and chemically (Figures 2c). For instance, a photocurable poly(ethylene glycol) diacrylate (PEGDA)-rich phase can form w/w emulsion droplets in the K_3PO_4-rich continuous phase (see methods in Figure S4 in the Supporting Information). Upon illumination of ultraviolet (UV) light, the PEGDA in the emulsion phase is cross-linked into hydrogel particles via free radical polymerization, as shown by the optical images in Figure 2a2. Under the scanning electron microscopy (SEM) analysis, the polymerized PEGDA particles exhibit dense microstructures, as shown in Figure 2a3.

Commonly used hydrogel precursors, such as the rat tail collagen, may not directly phase-separate with another water-soluble additive; nevertheless, the precursor molecules can be used as additives to a proven aqueous two-phase system, such as dextran–PEG–H_2O. This strategy requires the gel precursors to maintain a much higher local concentration in the droplet phase than in the continuous phase for subsequent gelation. The significant difference in the affinity of the precursors to the two immiscible aqueous phases, known as the partitioning effect, is widely found in macromolecular and colloidal precursors. As an example, when 1.5 mg/mL collagen is added into the mixture of dextran-in-PEG emulsion, more than 90 wt % of collagen is spontaneously enriched into the dextran-rich phase after phase equilibrium.[19] This partitioning effect allows formation of collagen microspheres with a highly porous microstructure by thermal gelation at 37 °C, as shown in panels b1–b3 in Figure 2. The resultant particles, which are composed of both dextran and collagen, can resist dilution in the 0.9 wt % saline solution. In comparison, without using the

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templates of w/w emulsions, droplets of collagen solution is only diluted in the PEG solutions.

Gelation of w/w emulsions can also be achieved by introducing chemical cross-linking of the droplets. Two reagents are separately added into the emulsion and continuous phases, and the reaction between them can be easily triggered since the w/w interface is highly permeable to water-soluble reagents. For example, sodium alginate and calcium chloride are separately added into the dextran-rich emulsion phase and the PEG-rich continuous phase in advance. After the formation of w/w emulsion droplets via all-aqueous electrospray, Ca ions can diffuse quickly across the w/w interface and react with alginate, yielding calcium alginate particles, as shown in Figure 2c. Because of the fast gelation, diffusion of alginate from the emulsion to the continuous phase is prohibited, so the gelation reaction is confined within the droplet phase. While the gelation reaction often induces undesired clogging in the microchannels, our AAE approach avoids this problem: before the emulsions form, reagents are separately dissolved in the droplet and continuous phases; subsequently, the chemical reaction starts only after the droplets are collected in the beaker with the continuous phase.

By using the w/w emulsions as templates, the volume of hydrogel particles scales with the volume of emulsion templates, and the final volumes of hydrogel particles are also affected by the diffusion of water across the w/w interface. When two almost equilibrated aqueous phases, for instance, a 15 wt % dextran solution and an 8 wt % PEG solution are used, the net osmosis across the w/w interface is negligible. Consequently, the volume of the resultant alginate particles is almost equal to that of the as-formed emulsion droplets. When the osmolarity of the emulsion phase is decreased by reducing the concentration of dextran from 15 wt % to 5 wt %, we observe that the resultant droplets shrink due to the diffusion of water from the emulsion to the continuous phase. Taking advantage of such an osmotic effect,[39,40] the final volume of the particles can be adjusted by varying the osmolarity of the two aqueous phases, as shown in Figure 3a. By labeling the dextran-rich phase with fluorescein isothiocyanate dextran (FITC-dextran), we find that dextran with a high molecular weight of 500 000 can be immobilized and encapsulated into the hydrogel particles, as demonstrated in the fluorescence microscope image in Figure 3b. The entrapment of water-soluble dextran in the hydrogel explains the increased osmolarity and reduced loss of water during gelation.

The all-aqueous electrospray represents a simple step for encapsulating bioactive proteins in hydrogel particles. As a model of bioactive protein, 1 wt % β-amylase is added into the emulsion phase of water with 10 wt % dextran and 1.5 wt % sodium alginate prior to the all-aqueous electrospray step. A dextran-free alginate solution (1.5 wt %) dissolved with 1 wt % amylase is used as the emulsion phase in the control group. Both emulsion phases are dispersed into the continuous phase of water with 8 wt % PEG and 1.5 wt % $CaCl_2$ to form particles. By using the dextran-in-PEG emulsion template, the encapsulation efficiency of the amylase is significantly increased from 30% ± 2% (in alginate particles) to 47% ± 3% (in dextran−alginate particles). The increased encapsulation efficiency is due to the enhanced partitioning effect of amylase to the dextran-rich emulsion phase, thus reducing the undesired leakage of amylase to the surrounding aqueous phase during fabrication. When transferred to a starch/PBS solution, the amylase is released from particles (see Figure S5 in the Supporting Information). In comparison to the fresh amylase solution, the amylase released from particles exhibits similar activity in digesting the starch (Table 1), suggesting the preservation of the encapsulated and released proteins by the proposed approach.

Since the AAE approach provides mild conditions for gelation of hydrogel particles, the cytocompatibility of this approach is further tested by encapsulating living cells. As a proof-of-concept, we encapsulate HEK 293 cells into the emulsion droplets and subsequently immobilize them into alginate particles. To avoid the death of cells induced by the osmotic effect, the osmolarity of the emulsion phase is adjusted to 320 mOsm/kg. The live/dead assay confirms that 76% ± 6% cells remain alive in the dextran/alginate composite particles, as shown by the fluorescence and bright-field microscope images in Figures 4a and 4b. Compared to the calcium alginate particles, the use of dextran-in-PEG emulsion templates does not cause reduction in the viability of HEK293 cells, as proved by the cytotoxicity assay in Figure 4c. These observations suggest the highly cytocompatible nature of the AAE approach.

3.3. Water-in-Water-in-Water (w/w/w) Double Emulsion Templated Capsules. Using all-aqueous emulsions with advanced structures enables versatile control over the interior architectures of hydrogels. The ability to tailor the emulsion structure in the all-aqueous emulsion system, such as w/w/w double emulsions, will have important implications for biochemical and biomedical applications of hydrogel microspheres. Previously, we demonstrate a phase separation approach for producing w/w/w double emulsions in the microfluidic channels.[36] Briefly, a single-phase solution containing 5 wt % dextran and 1 wt % PEG (23 mOsm kg^{-1}, measured by an osmometer (Model 3320, Advanced Instrument, Inc., USA)) is dispersed into an 8 wt % PEG-rich continuous phase (60 mOsm kg^{-1}) to form single emulsions. Because of the higher osmolarity in the 8 wt % PEG-rich continuous phase, water is extracted from the emulsion phase, concentrating the two incompatible solutes inside the droplets. Once the solute concentrations exceed a threshold, the droplet separates into a shell of the dextran-rich phase and inner core droplets of the PEG-rich phase, as shown in the schematic in Figure 5a. The viscous dextran-rich shell prolongs or even prohibits the escape of PEG-rich inner droplets out of the shell. However, inside the dextran-rich shell, the PEG-rich inner droplets still merge with each other, until only one inner droplet is left in each double emulsion droplet, as shown by the phase-contrast microscope image of the double emulsion in Figure 5b.

Immobilization of the double emulsion structure formed at the different time points of phase separation enables engineering of the internal structures of the capsules. This creates new opportunities in the mimicking of the compartmentalized cellular structures[41] and the kinetic regulation of the biochemical reactions in the droplet-based bioreactors.[42] To immobilize the double emulsion structure, 1.2 mg/mL collagen is initially loaded onto the droplets before the start of phase separation. After phase separation occurs, collagen spontaneously gets enriched in the dextran-rich shell phase (Figure 5c). By adding the cross-linker of collagen from the continuous phase, the emulsion structure can be quickly immobilized (see Figure S6 in the Supporting Information). At the early stage of phase separation, fast gelation of the shell leads to the formation of capsules encapsulating multiple compartments of PEG-rich droplets with a polydisperse size distribution, as

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demonstrated in the microscope image in Figure 5d. With the coalescence of these PEG-rich inner droplets, the number of these droplet chambers reduces; correspondingly, the average diameter of these compartments increases (Figure 5e). After the complete coalescence of the PEG-rich inner droplets, gelation of the collagen-rich shell results in one big droplet chamber enclosed in each capsule (Figure 5f). Because of the partitioning of collagen in the dextran-rich shell, the diameter ratio of the inner core to the entire droplet decreases from 0.76 ± 0.05 to 0.62 ± 0.04.

All-aqueous double emulsions can be used for the histologic examination and separation of diseased amyloid proteins from healthy collagen fibers. Amyloid fibrils are known as misfolded protein fibers, the presence of which correlates with many diseases, such as neurodegenerative disorders.[43] These diseased proteins are deposited in the extracellular matrices and mixed with collagen fibers, making it difficult to be filtered and detected. Amyloid disease is traditionally diagnosed by staining of the pathological section. This method requires tedious steps of fixation, embedding, and sectioning of the samples before staining. Moreover, the diagnosis can hardly be made by staining alone if the amyloid fibrils are sparsely distributed or their volume fraction is small. Here, we use a mixture of collagen fibers and amyloid fibrils to mimic the diseased extracellular matrices. To separate the amyloid from collagen, the protein mixture is first suspended into the droplet phase of 5 wt % dextran and 1 wt % PEG (pH 4), as illustrated schematically in Figure 6a. After the phase separation of the droplets in the 8% PEG solution, we find that collagen becomes enriched in the shell while amyloid fibrils constitute the inner core and occupy the droplet interface. This is proved by labeling the lysozyme fibrils with a fluorescence dye, thioflavin T (ThT), in Figures 6b–e. The separation is guided by the affinity of different proteins to different phases of the double emulsions. After the gelation of the double emulsion, the amyloid fibrils are locally immobilized and preserved in the microcapsules. The level of the amyloid proteins can be quantitively determined by measuring the relative intensity of fluorescence in the core and shell phases of the double emulsion, as shown in Figure 6f. When compared with the traditional method of histological staining, our approach is easy to operate and only requires a tiny amount of tissue samples (10^{-9}–10^{-8} g).

4. CONCLUSION

All-aqueous emulsion droplets are used as novel templates for controlling the formation of hydrogel particles and capsules. To control the size and structures of hydrogel particles, we first introduce an all-aqueous electrospray approach to generate monodisperse all-aqueous emulsion droplets, and the subsequent solidification of these emulsion droplets yields hydrogel particles and capsules. The sizes and structures of the hydrogel materials can be adjusted by controlling the shape of the all-aqueous emulsion templates.

While traditional techniques for making hydrogel capsules often utilize water-in-oil emulsion droplets or solid substrate materials as templates, the use of all-aqueous emulsion templates provides a biocompatible choice to shape the microgels. This approach not only simplifies the traditional protocols of preparing hydrogel capsules, but also avoids the denaturation of protein and the loss of cell viability due to direct contact with water/oil interfaces or organic solvents. Therefore, the use of all-aqueous emulsion droplets as templates is promising for many biomedical applications, especially for the assembly of living colloidal species,[44] transplantation of cells,[45] and fabrication of tissue scaffolds.[46]

■ ASSOCIATED CONTENT

Supporting Information

Transition of the flow regimes in the all-aqueous electrospray, analyzed as a function of the applied voltage (Figure S1). Details on the jetting regime in the all-aqueous electrospray (Figure S2). All-aqueous electrosprayed fabrication of PEGDA emulsion droplets (Figure S3). A calculation of the Oh numbers when droplets are formed through the AAE approach and the droplet-based microfluidic approach (Figure S4). Release of bioactive amylase from the dextran–alginate composite particles (Figure S5). Details on the fast immobilization of the double emulsion structures (Figure S6). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b02708.

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Notes

The authors declare no competing financial interest.

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