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One-Step Synthesis of Hyaluronic Acid-Based (Sub)micron Hydrogel Particles: Process Optimization and Preliminary Characterization

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Hyaluronic acid (HA)-based (sub)micron hydrogel particles were synthesized by crosslinking virgin HA with divinyl sulfone (DVS) in sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micelle systems under basic conditions. Experimental parameters including HA molecular weight, solution concentration, and reaction time were systematically varied in order to obtain well defined hydrogel particles. The use of a cosurfactant, 1-heptanol (1-HP), had a profound effect on the reaction kinetics and the particle morphology. The resulting particles exhibit negative charges on their surfaces. In vitro biocompatibility study indicates that these particles do not cause severe death to the cultured fibroblasts. These HA-based hydrogel particles are promising candidates for use in drug delivery.

Key Words: Hyaluronic acid; (Sub)micron particles; Drug delivery; Biomaterials; Biodegradable polymer; Microgel; Nanogels

Introduction

Hyaluronic acid (or hyaluronan, HA) is a naturally occurring linear polyelectrolyte based on repeating disaccharides of β1-4 D-glucuronic acid and β1-3 D-*N*-acetylglucosamine ($[\beta$-1,4-GlcUA-β-1,3-GlcNAc$]_n$). It is known to be the only non-sulfated glycosaminoglycan in the extracellular matrices (ECM) of all higher animals.[1] It not only regulates the ECM organization, but also plays vital roles in cell adhesion, cell motility, and cancer metastasis.[2] It is essential for morphogenesis and embryonic development.[3] It is indispensable for growth factor action, cartilage stability, and joint lubrication.[4,5] In addition to its intriguing biological functions, HA's inherent biocompatibility and biodegradability makes it an attractive biomaterial in drug

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delivery[6,7] and tissue engineering.[8−10] Its short lifetime (varies from minutes to weeks depending on the type of tissue),[11] however, necessitates chemical modifications and physical processing to improve its stability.

HA-based hydrogel particles are attractive materials for biomedical applications because they can provide enormous surface area that will improve tissue integration and facilitate drug delivery. Multifunctional HA hydrogel particles can be engineered to present therapeutic factors at targeted locations and deliver them in a controlled fashion.[12] A variety of particles can be synthesized using the unique micro-environment of the emulsion system.[13,14]For the preparation of HA hydrogel particles, generally a chemical modification is necessary to impart functionality to HA prior to the particle synthesis.[12,15,16] Hoffman et al. demonstrated the chemical crosslinking of HA in bulk using divinyl sulfone (DVS) and viniyl sulfone-poly(ethylene glycol)- vinyl sulfone (VS-PEG-VS) as crosslinkers, and the resulting hydrogels were used to deliver anti-inflammatory drugs.[17] HA crosslinked by DVS has been used to treat osteoarthritis.[18,19] However, this chemistry has not been successfully utilized to synthesize HA hydrogel particles with diameters in the (sub)micron range. Here, we report the synthesis of HA (sub)micron hydrogel particles using a water-in-oil microemulsion system as the template and DVS as the crosslinker. We systematically varied the reaction time, the molecular weight and solution concentration of HA, and the relative concentration of the co-surfactant in order to obtain the optimum conditions for particle synthesis. Throughout the study, the water-to-surfactant molar ratio (W_0) was kept constant.

Experimental Section

Materials

Hyaluronic acid (varying molecular weight) was obtained from Lifecore Biomedical and Genzyme Corp. Divinyl sulfone (DVS, >98%), sodium bis(2-ethylhexyl)sulfosuccinate (AOT, 98%), and 2,2,4-trimethylpentane (isooctane, 99.8%) were obtained from Sigma-Aldrich Chem. Co. 1-heptanol (1-HP, 99%) was obtained from Alfa Aesar. NIH/3T3 fibroblasts (ATCC, CRL-1658) and the MTT viability assay kit (ATCC, 30-1010K) were purchased from ATCC. Cell culture media and the supplements were obtained from Invitrogen. All chemicals and reagents were used as received.

Synthesis of HA (sub)micron particles

HA (sub)micron particles were synthesized using a water-in-oil microemulsion system. In a typical experiment, 0.54 mL of HA solution (6.8 wt% in 0.2 M NaOH, HA MW: 560 kDa) was dispersed in 15 mL isooctane containing 0.2 M AOT and 0.04 M 1-HP. The mixture was vortexed until a clear solution was obtained. DVS (60mol% relative to the HA repeating unit) was subsequently added, and the reaction was allowed to proceed for 1 h at ambient temperature under continuous mixing with a stirring speed of 2000-2200 RPM. The reaction mixture was subsequently filtered into acetone using a Whatman filter paper (particle retention >8 μm). The top organic phase (isooctane and acetone) was decanted, and the precipitate was re-suspended in acetone and collected by centrifugation at 4500 rpm for 10 min. For the 15 mL AOT solution containing HA particles, 200 mL of acetone was used. The yield was over 90% measured gravimetrically. The particles were subjected to repeated washing (at least 3 times) with acetone. Particles were collected by centrifugation and dried under vacuum at 25 °C. For separation purposes, the reaction mixture was subjected to a second filtration (particle retention >2.5 μm) before being collected in large excess of acetone.

Characterization of HA Particles

Morphology

HA hydrogel particles were imaged using a Field Emission Scanning Electron Microscope (SEM, JSM-7400F) with an operating voltage of 1-3 kV. The powdery HA hydrogel particles were deposited on an aluminum SEM stub and coated with gold on a Denton Sputter Coater for 45 s. To prepare samples for Transition Electron Microscope (TEM) imaging, a drop of reaction emulsion was placed on the copper grid and subsequently stained with uranyl acetate followed by solvent evaporation. An operating voltage of 200 KeV was applied using JEOL 2000fx TEM with a LaB_6tip.

Surface charge and particle size distribution

Zeta potential measurements were carried out using Malvern Zetasizer (Nano Series) (Model # ZEN 3600). HA particles were dispersed in distilled water prior to the measurement. Particle size analysis was performed with dynamic light scattering measurements (DLS) using Malvern Particle Size Analyzer (Brookhaven Ins. Corporation) by suspending HA particles in 10 mM KCl solution and the result is the average of 3 consecutive measurements.

Biocompatibility

The biocompatibility of the HA particles was assessed using NIH 3T3 fibroblasts at passage 3. Cells were seeded onto 24-well plates at a density of 40,000 cells/well in 1 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 1% penicillin-streptomycin. HA particles were sterilized by UV irradiation for 30 min in a sterile tissue culture hood that is equipped with a UV lamp. The sterilized particles were subsequently diluted with the medium and the final particle concentration was in the range of 0.002-2.0 mg/mL. The relative toxicity was assessed with a commercially available MTT viability assay kit (ATCC) after 2.5 days of culture without changing the medium. The solution absorbance was read at 570 nm (PekinElmer Universal Microplate Analyzer) after the particles were removed. Results are reported as the average (n = 5) and standard deviation of the measured absorbance normalized to the absorbance of NIH/3T3 cells cultured in unmodified media.

Results and Discussion

Synthesis of HA hydrogel particles

HA-based hydrogel particles are attractive biomaterials for use as drug delivery vehicles and tissue engineering scaffolds. We are interested in developing a simple process that allows for efficient synthesis of HA hydrogel particles. Previous studies on the synthesis of HA particles require chemical modification and extensive purification to prepare HA derivatives with reactive groups.[12,15] Although a wide range of chemical modification methods are available for this purpose[25−28], the synthesis and purification procedures not only are tedious but also reduce the overall yield. Usually, particles thus synthesized are well beyond micron size range.[20] We have developed a 1-pot approach using the reverse micelle crosslinking technique. AOT was chosen to stabilize the reverse micelle in an isooctane continuous phase since the synthesis of hydrogel particles in AOT reverse micelle system has been successfully demonstrated.[21−23] Figure 1 is a schematic

illustration of the AOT reverse micelles that have water pools confined within the surfactant molecules. The reverse micelles were used as the micro reaction vessels for the synthesis of HA-based hydrogel particles. Several experimental parameters need to be defined before we discuss the synthetic procedures. W_0 (W_0= $[H_2O]/[AOT]$) describes the relative amount of water and surfactant used in the water-in-oil microemulsion system[23], where the water droplets containing HA (aqueous phase) were suspended in a continuous oil phase (isooctane). Co-surfactant, such as 1-heptanol (1-HP) can be used to stabilize the emulsion and control the particle size. The molar ratio of AOT and 1-HP is defined as R_m (R_m = [AOT]:[1-HP]). The amount of DVS relative to HA repeating units is defined as X%. The reaction was performed under continues mixing (2000-2200 RPM); upon addition of DVS, the water droplets containing HA molecule collided with DVS molecules which has substantial water solubility. The crosslinking reaction proceeds via the Michael type addition that results in the formation of a 3D micro-network.[24] The DVS chemistry employed here eliminates the need to prepare HA precursors since DVS can directly react with the hydroxyls on HA backbone in basic conditions.

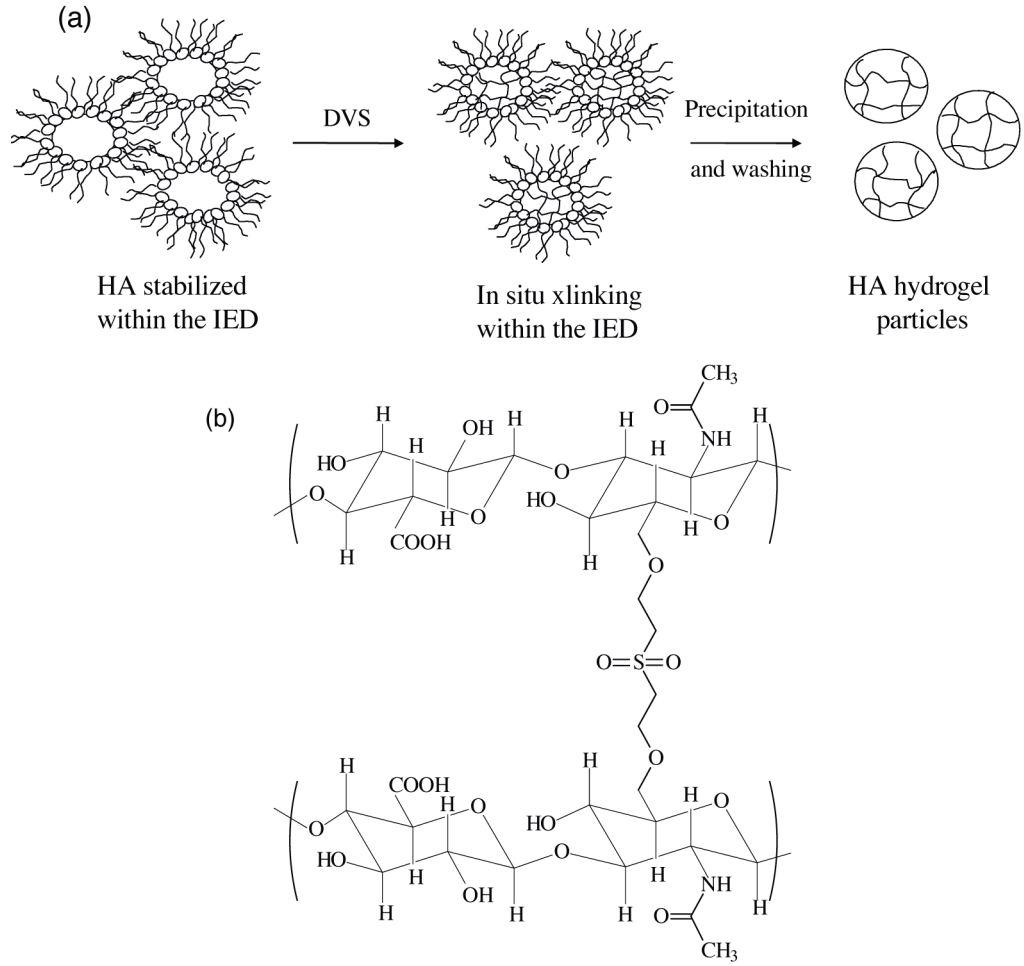

Figure 1. Schematic illustration of HA particle synthesis in AOT reverse micelles (a) and chemical structure of DVS crosslinked HA (b) IED: Inverse emulsion droplets.

The effect of HA concentration

To investigate the effect of HA concentration on particle synthesis, emulsions containing different amounts of HA were prepared while keeping the W_0 value constant. HA was first dissolved in 0.2 M NaOH solution before being added to the continuous phase. The alkaline condition is required for covalent crosslinking of HA with DVS. An added advantage of this reaction condition is the dramatic decrease in solution viscosity, leading to relatively stable reverse micelles. Previous study has shown that only limited HA degradation was observed in basic medium (pH 12.6) .[29]. In this study, a typical W_0 value of 10 was used for particle synthesis.[22] Acetone was chosen as the solvent to remove the surfactant and the un-reacted crosslinker because it not only destabilizes the AOT-coated micelles but also precipitates HA hydrogel particles. The washing procedure was repeated at least 3 times. Table 1 summarizes the reaction conditions evaluated. In all experiments, the molecular weight of HA (Mw = 560 kDa), the amount of DVS relative to HA repeating units (X% = 60 mol%), and water-surfactant molar ratio (W_0) were kept constant (W_0 = 10), while the HA concentration was systematically varied. Figure 2 shows the SEM images of HA hydrogel particles prepared after a 4-h reaction for run #1 and run #3 in Table 1, respectively. Run #1 led to particles with varying sizes embedded in a continuous matrix (Figure 2a). Although smaller particles can be obtained with run #3, they have ill-defined shapes. These particles do not disperse readily in water, suggesting that they may be interconnected to form large aggregates. We suspect that a 4 h reaction may not be sufficient to obtain discrete particles with perfectly spherical shapes. In addition, the amount of HA in the aqueous solution should be above certain critical concentration in order to obtain particles with well defined morphology. Obviously, if the HA concentration is too low, effective crosslinking within the micelles cannot be established. Thus, it was decided that a longer reaction time and a minimum amount of 0.54 mL HA solution (6.8% in 0.2 M NaOH) are necessary. We elect to keep W_0 constant throughout the studies.

Table 1. Reaction conditions for the synthesis of HA particles using different amount of HA (M_W: 560 kDa; reaction time: 4h).

Run number	C_{HA}(wt%)[1]	V_{DVS} (μL)[2]
1	6.8	5.6
2	5.1	4.2
3	3.4	2.8
4	1.7	1.4

1. HA weight percent concentration in 0.2 M NaOH solution;
2. Amount of DVS in μL added.

The effects of reaction time and HA molecular weight

To investigate the effect of reaction time on the particle formation, reaction were terminated at different times (1, 3, 5, and 12 h) while HA molecular weight (560 kDa), W_0 (W_0=10) and X% (X% = 60 mol%) remained the same. The SEM images of the resulting particles are summarized in Figure 3. Discrete particles were obtained only when the reaction mixture was stirred for 12 h as shown in Figure 3 (d). Again, shorter reaction times resulted in particles embedded in a continuous matrix, indicating an incomplete reaction. Figure 4 shows the SEM images of hydrogel particles obtained with HA molecular weight of 1360 kDa (Figure 4a), 560 kDa (Figure 4b), and 169 kDa (Figure 4c). The reaction was carried out for 12 h at

ambient temperature. Again, X% and W_0 were kept constant regardless of HA molecular weight. When 1360 kDa HA was used, big chunks of irregular particles were observed (Figure 4a), suggesting unstable micelles and/or incomplete crosslinking within the reverse micelles. It is hypothesized that the conformation and partitioning of the HA within the reverse micelle determine the morphology of the resulting hydrogel particles. HA with higher MW adopts a more compact conformation within the reverse micelles, thereby leading to limited accessibility by DVS. Furthermore, high MW HA leads to higher solution viscosity, making it difficult to stabilize the reverse micelles under the same condition. As a result, there is a greater chance for crosslinking from the surface of the reverse micelles. Under the continuous mixing conditions, intra-micellar crosslinking (inside the reverse micelles) takes place simultaneously with inter-micellar crosslinking (among the reverse micelles) because the droplets are dynamic entities. Evidently, intra-micellar crosslinkings are more rapid because of the stabilization effect by the surfactants and the concentration of the reacting species (water pools are enclosed with the surfactant as shown Figure 1). However, during droplet collisions, inter-droplet polymerization does occur, resulting in bigger particles with wide size distribution and irregular morphology. Although one can obtain discrete spherical particles (Figure 4c) with low molecular weight HA (169 kDa), only a small portion of the final product has well-defined spherical morphology. Polymer chains of low MW HA may not be long enough for effective crosslinking within the micelles. Therefore, for the optimal HA hydrogel particle preparation, the MW of HA was chosen to be around 500 kDa.

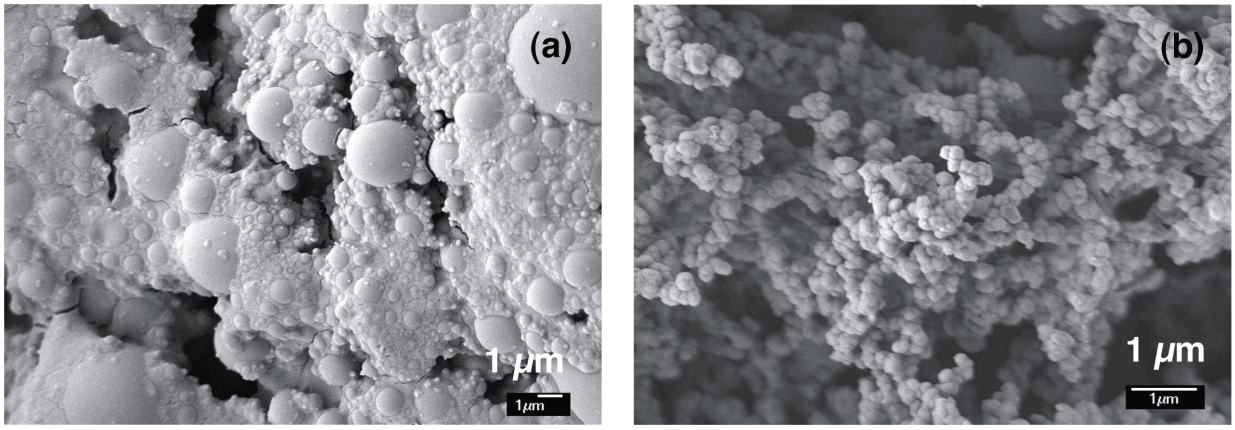

Figure 2. SEM images of HA hydrogel particles synthesized after 4 h reaction for run #1 and #3 from Table 1, respectively.

The effect of co-surfactant

Above the critical concentration, AOT surfactant forms stable reverse micelles containing water droplets in oil phase, these droplets or water pools (reverse micelles) can be used as reactor for particle synthesis. It was reported that the addition of co-surfactant to AOT micellar system has a great effect on the surfactant solvation kinetics[30]. When 1-heptanol is used as a co-surfactant, HA solutions with higher W_0(>10) can be prepared. To ensure homogenous exchange of reactive species between micelles that are in a dynamic equilibrium, 1 h reaction was performed for different molar ratios of AOT to 1-heptanol (R_m = [AOT]:[1-HP]), keeping W_0=10. Figure 5(a) illustrates the chemical structures of the surfactant and co-surfactant. Figure 5(b), (c), and (d) are the SEM images of the particles prepared after 1 h reaction using R_m value of 5/1, 5/3, and 5/5. It was found that the co-surfactant has a significant effect on the reaction kinetics. The

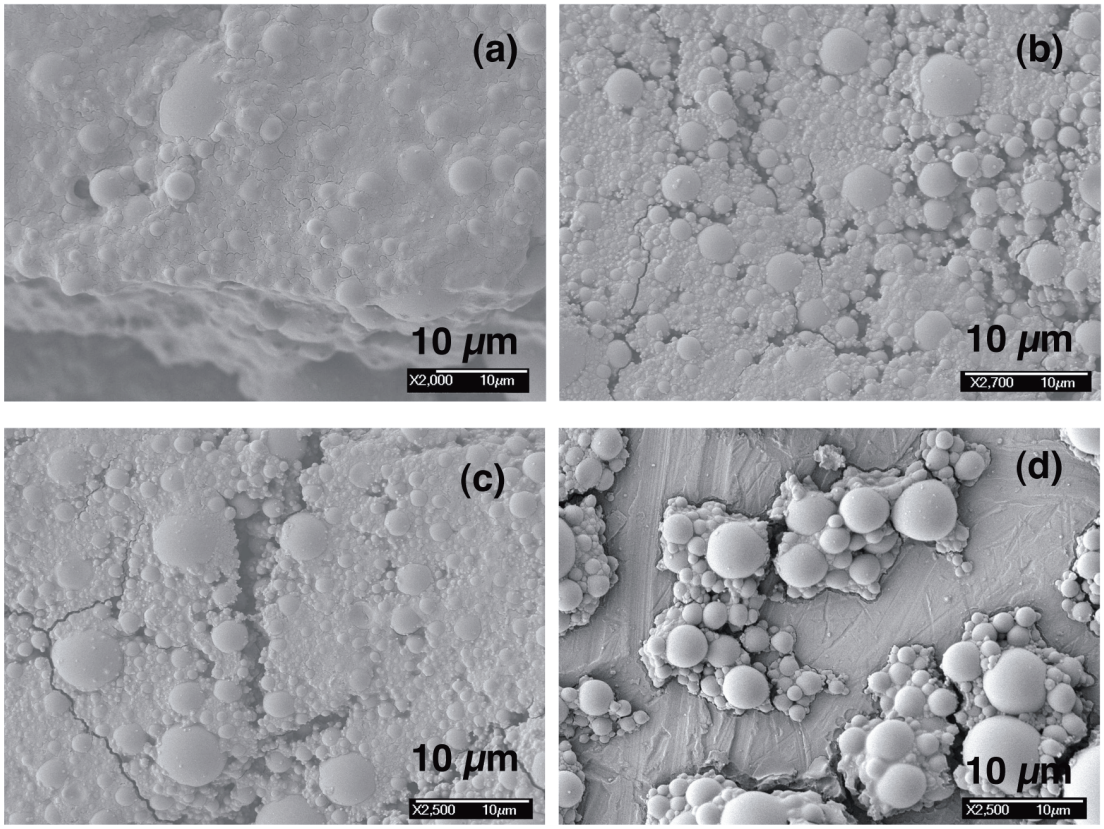

Figure 3. SEM images of HA hydrogel particles obtained with different reaction times: 1, 3, 5, and 12 h for (a), (b), (c) and (d), respectively. (M_W: 560 kDa and W_0:10).

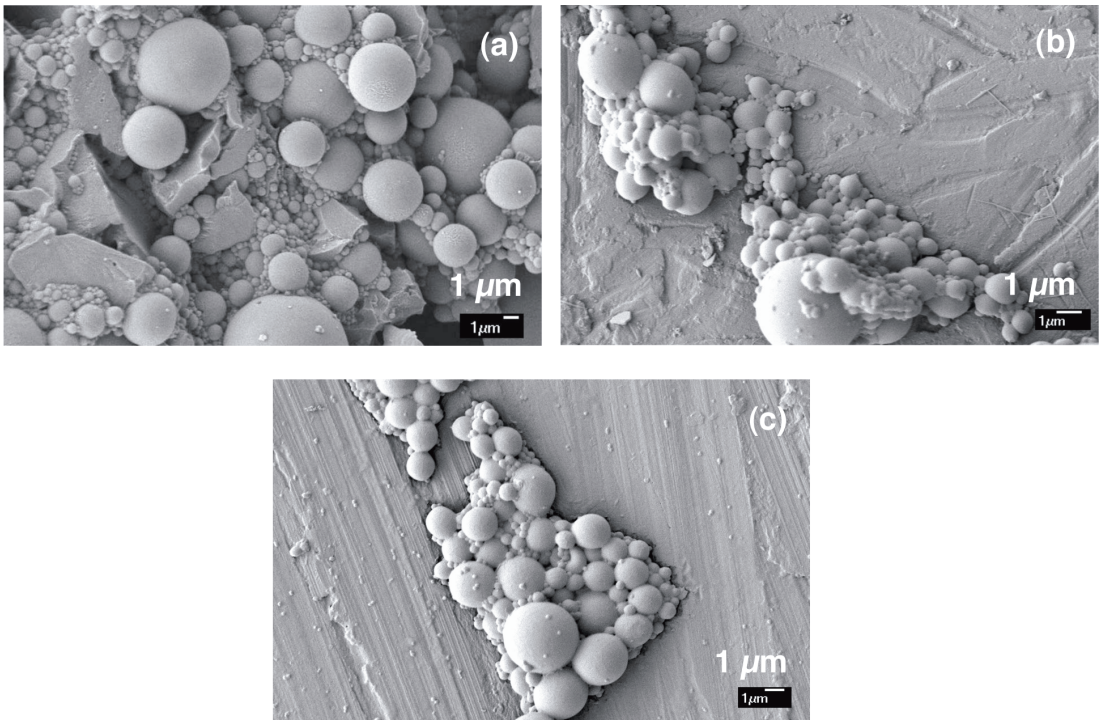

Figure 4. SEM images of HA hydrogel particles obtained using HA with different molecular weights: 1.36 MDa, 560 kDa, and 169 kDa for (a), (b) and (c), respectively (reaction time: 12 h; W_0:10).

reaction time was greatly reduced (from 12 h to only 1 h), yielding particle with well defined spherical shape. As the amount of 1-HP increases, the particles become bigger, as seen in Figure 5(d). Although particles obtained from R_m = 5 (Figure 5b) and 1.67 (Figure 5c) are similar in terms of their size and morphology, R_m of 5 requires the minimum amount of co-surfactant. From these studies, we conclude that R_m of 5 and a reaction time of 1 h is optimum for the preparation HA (sub)micron hydrogel particles. Figure 6 shows the SEM images of particles prepared after a 12-h reaction with R_m values of 5, 5/3, and 5/5 for (a), (b) and (c), respectively. Particle morphology and size did not change dramatically as the reaction time was increased. Thus, we conclude that an R_m of 5 and reaction time of 1 h is the best condition for the preparation of HA (sub)micron particles (at W_0=10) from 6.8wt% HA solution in 0.2 M NaOH with 60 mole % DVS as a crosslinker. The presence of co-surfactant maybe useful for HA particle synthesis from high MW HA; however, for HA with MW around 500 kDA, the existence of co-surfactant does not significantly affect the HA particle synthesis in this method. We realize that SEM images alone do not allow us to determine whether the reaction is complete or not. They, however, can provide critical insight for the successful synthesis of HA hydrogel particles with defined shapes.

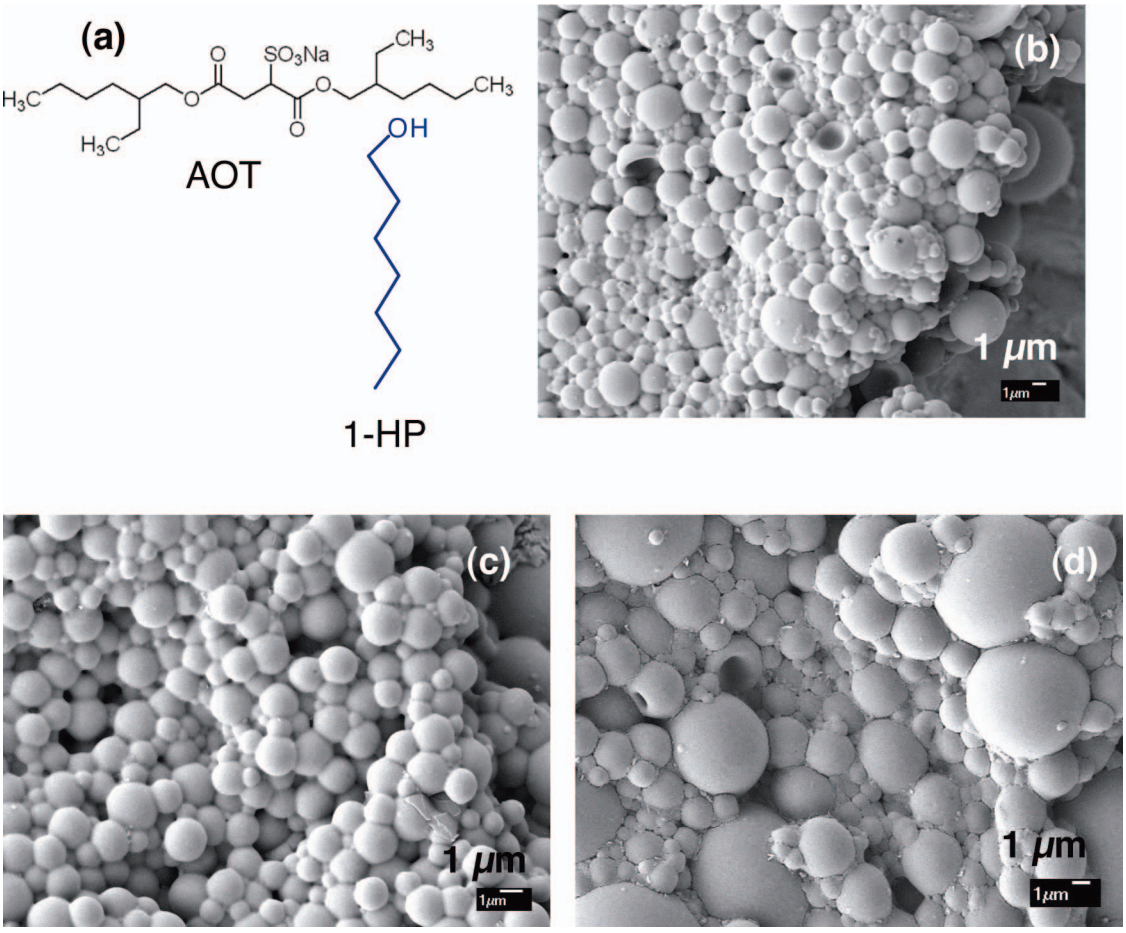

Figure 5. Chemical structures of the surfactant (AOT) and the cosurfactant (1-HP) (a), and SEM images of HA particles synthesized with different R_m values: 5/1, 5/3, and 5/5 for (b), (c), and (d) respectively. (reaction time: 1 h; W0:10).

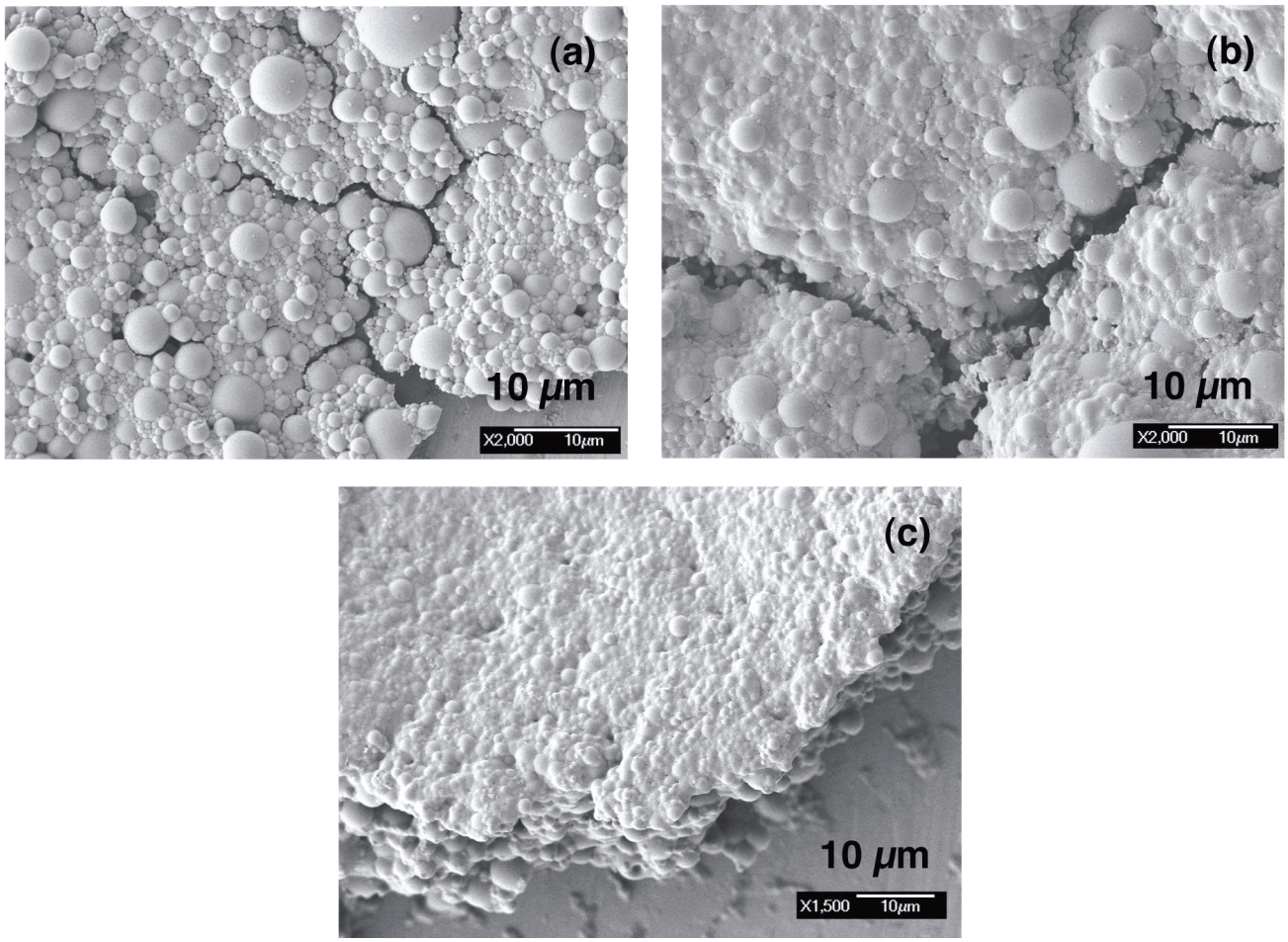

Figure 6. SEM images of HA particle synthesized with different R_m values: 5/1, 5/3, and 5/5 for (a), (b) and (c), respectively (Reaction time: 12 hr).

Despite the broad size distribution of HA particles obtained by the AOT reverse micelle system, particles with narrower size distribution can be readily obtained by filtration. To this end, the reaction emulsion was poured over a second filtration paper with a pore size smaller than 2.5 μm. The high yield of the initial synthesis, combined with the simple filtration step, offers convenient process to obtain particles of submicron dimension. Fig. 7 shows the SEM images of particles prepared using the best reaction condition before (7a), and after (7b) additional filtration using filtration membrane with particle retention of >2.5μm. It is obvious that smaller particles can readily be separated. Figure 7(c) shows the TEM image taken on the emulsion droplets directly from the reaction mixture prior to the precipitation. It clearly confirms the presence of nano-sized reverse micelles that are filled with HA.

Characterization of HA hydrogel particles

When using particles for biomedical applications, surface charge[31,32] and surface functionality[33,34] are very important. Therefore, HA particles were dispersed in distilled water, and the zeta potential measurements were carried out. Figure 7d demonstrates negatively charged surfaces with the zeta potential value of ca.

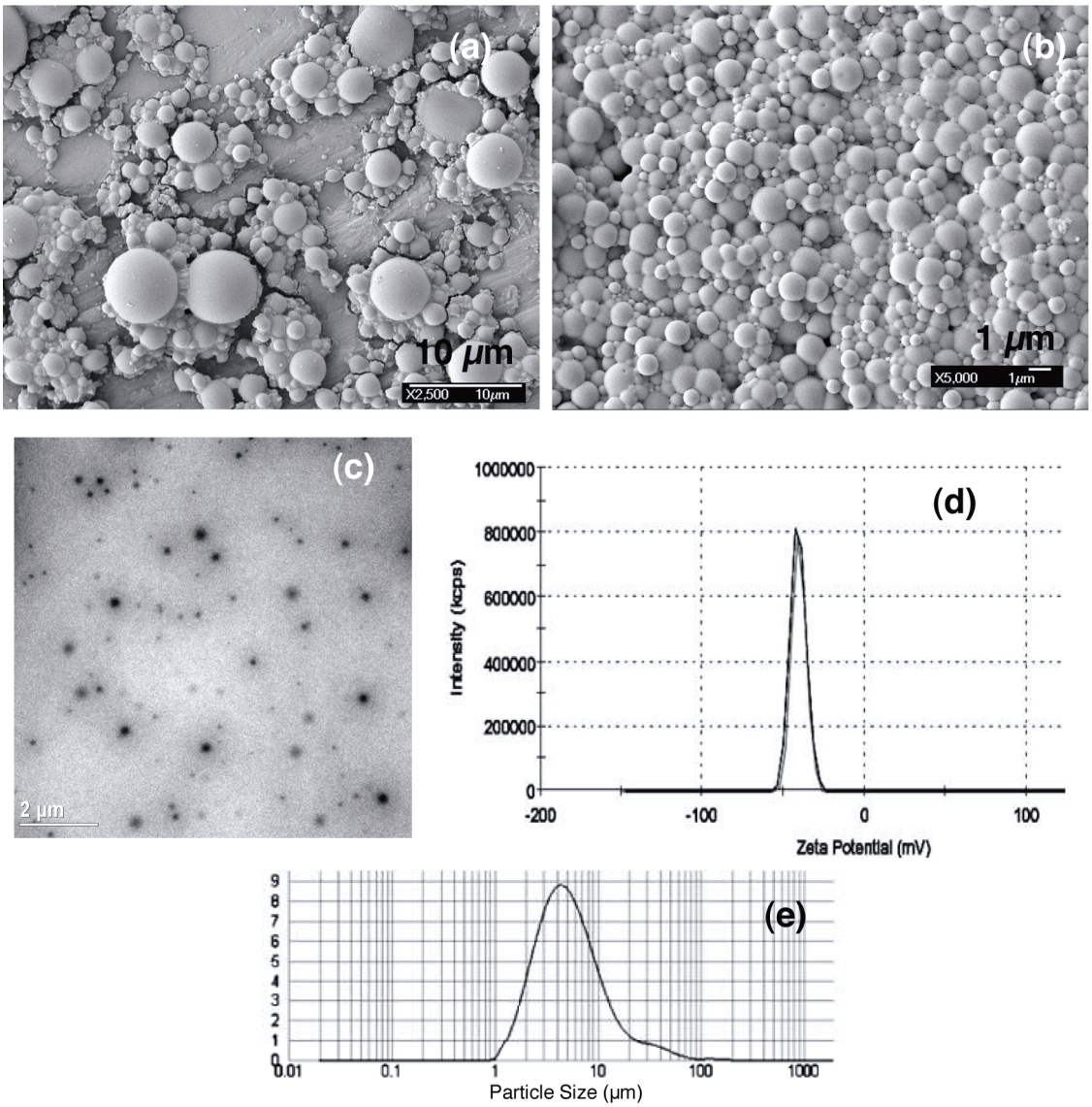

Figure 7. SEM images of HA particles before (a) and after (b) membrane filtration (particle retention >2.5 μm). Reaction conditions: 1 h reaction, Rm = 5/1. (c) TEM image of the emulsion taken directly from the reaction mixture after 1 h of reaction without any filtration. (d) Zeta-potential measurement of HA particles disperse in distilled water. (e) Particle size distribution of HA particles in 10 mM KCl (MW:419 kDa).

–40 mV, indicating the preservation of the carboxylic acid functionality on HA after particle formation. Although the presence of residual surfactant molecules (AOT) on the particle surface can contribute to the observed negative zeta potential, we believe the repeated acetone wash and water effectively removed most of the surfactant. On the other hand, washing with distilled water can cause the loss of nanosized particles, as can be shown in Figure 7e, which illustrates particle size distribution of HA particles after thoroughly washing with acetone and water. Particles were collected with 4500 rpm centrifugation and the average size found to be 4.88 μm. The tailing in the figure can be attributed to the impurities since no filtration was performed before DLS measurements. In contrast to the multi-step HA particle preparations that require the introduction of functional groups to HA via the COOH group, our method takes advantage of the natural

reactivity of the OH groups towards the crosslinker. The DVS amount was chosen to be 60 mol% relative to the HA repeating unit since previous work by Hoffman et al. indicated that this concentration leads to strong hydrogels in bulk.[17] The residual –OH groups present on the particles can be utilized for further modifications. In a separate study, we are investigating the potential of post modification to fine-tune the material properties.

In vitro cell cytotoxicity study is the first step in evaluating the potentials of the HA-based hydrogel particles for biomedical applications. Figure 8 shows the relative viability of NIH 3T3 fibroblasts after incubation with filtered HA particles for 2.5 days as a function of particle concentration. Particles were sterilized by direct UV exposure using the UV lamp inside a biosafety cabinet for 30 min and were subsequently cultured in direct contact with the cells. Cells cultured in the absence of HA particles were used as the control, and the measured absorbance is normalized to the absorbance of the control sample. A slight decrease in cell viability was observed when the particle concentrations increased from 0.002 to 2 mg/mL. Over 80% viability was observed at high concentrations. These results indicate that these hydrogel particles do not elicit severe cell death. The toxicity may be contributed to the impurity in the particles that could leach out during prolonged culture. More thorough purification may be necessary. Our initial degradation studies indicate that these hydrogel particles are extremely stable against hyaluronidase, the enzyme that is responsible for much of the HA catabolism in the organisms.[11] No degradation was detected within 24 days of incubation in the presence of 100U/ml hyaluronidase.

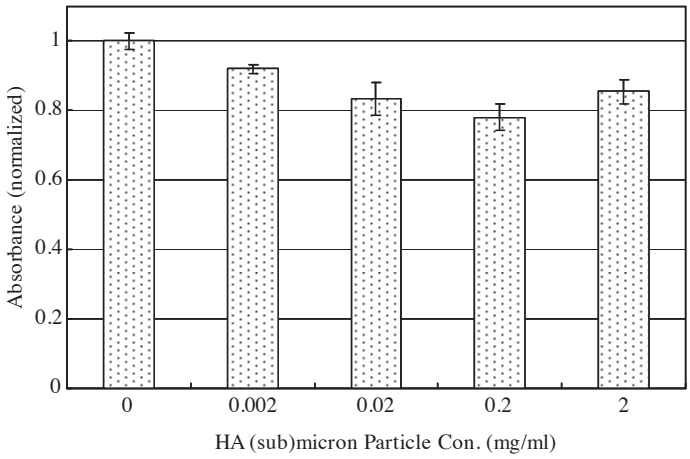

Figure 8. Viability of NIH 3T3 fibroblasts cultured for 2.5 days in the presence of HA particles as measured by an MTT viability assay. The measured absorbance is normalized to the absorbance for the control group. The control group corresponds to the assay just with cells in culture medium.

We have demonstrated that HA (sub)micron hydrogel particles can be prepared using DVS as the crosslinker via a single-step process. Chemical modification after particle formation can be used to introduce other functional groups such as aldehyde, hydrazide, and double bonds. These groups can be used as a reactive handle for bioconjugation.[6,35,36] We anticipate that drug molecules can be covalently anchored on the particle surfaces or physically encapsulated inside the particles, resulting in different release profiles. The release kinetics can be tuned by exploiting the conjugation chemistry, particle size, and crosslinking chemistry. This is the subject of our current investigation, and the results will be reported in the future.

Conclusions

We have developed a simple process for the synthesis of HA hydrogel (sub)micron particle using DVS as the crosslinker and a water-in-oil microemulsion system as the template. Through systematic adjustment of the experimental parameters, we found that the optimum reaction conditions require: (1) HA molecular weight around 500 kDa; (2) HA concentration in 0.2 M NaOH be 6.8 wt%; (3) W_0 value of 10; (4) reaction time of around 1 h; (5) addition of co-surfactant (1-HP) with an R_m value of 5. This technique is highly efficient and practical. It does not require tedious modification and purification steps for the introduction of reactive groups to HA prior to particle synthesis. The resulting HA (sub)micron hydrogel particles do not cause severe cell death, and HA's natural functional groups (–OH, COOH) are still available for further reaction. Additionally, this method provides HA particles that can be separated easily according to their size by simple filtration and centrifugation.

Acknowledgement

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