Flexible control of cellular encapsulation, permeability, and release in a droplet-templated bifunctional copolymer scaffold

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Designing cell-compatible, bio-degradable, and stimuli-responsive hydrogels is very important for biomedical applications in cellular delivery and micro-scale tissue engineering. Here, we report achieving flexible control of cellular microencapsulation, permeability, and release by rationally designing a diblock copolymer, alginate-conjugated poly(N-isopropylacrylamide) (Alg-co-PNiPAM). We use the microfluidic technique to fabricate the bifunctional copolymers into thousands of mono-disperse droplet-templated hydrogel microparticles for controlled encapsulation and triggered release of mammalian cells. In particular, the grafting PNiPAM groups in the synthetic cell-laden microgels produce lots of nano-aggregates into hydrogel networks at elevated temperature, thereafter enhancing the permeability of microparticle scaffolds. Importantly, the hydrogel scaffolds are readily fabricated via on-chip quick gelation by triggered release of Ca^{2+} from the Ca-EDTA complex; it is also quite exciting that very mild release of microencapsulated cells is achieved via controlled degradation of hydrogel scaffolds through a simple strategy of competitive affinity of Ca^{2+} from the Ca-Alginate complex. This finding suggests that we are able to control cellular encapsulation and release through ion-induced gelation and degradation of the hydrogel scaffolds. Subsequently, we demonstrate a high viability of microencapsulated cells in the microgel scaffolds. Published by AIP Publishing. [<http://dx.doi.org/10.1063/1.4972107>]

INTRODUCTION

Smart hydrogel microparticles have been increasingly emerging as highly important biomaterial scaffolds for many valuable applications including drug delivery and tissue engineering.^{1–4} Various hydrogels including agarose, poly-ethylene (PEG), collagen, and alginate have been widely used as extracellular matrixes (ECMs) for cell encapsulations.^{5–7} For instance, natural polymers are highly biocompatible upon mild gelation by temperature or Ca^{2+} , but they cannot enable an excellent permeability of large substances such as enzymes, antibodies, and leucocytes owing to immune defense.^{8,9} Synthetic polymer matrixes (e.g., PEG, Poly(N-isopropylacrylamide) (PNiPAM), etc.) are chemically and physically controlled as required properties such as morphology and permeability, but they usually harvest harmful radicals by rapid cross-linking via exposure to UV light.^{10,11} In many cases, the development of bio-degradable scaffolds is also greatly required for basic biomedical researches.^{12,13} Despite recent efforts, however, there is still lack of capability to simultaneously control the chemical and physical properties for cell growth and stimuli.¹⁴ For these reasons, it has been attracting intense interest and also challenged by the combination of penetrative, cell-compatible, and biodegradable properties for the cell microenvironment.

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Fabrication of micrometer-sized polymeric microparticles will enable to construct precisely controlled 3D-scaffolds and cellular microenvironment for embedded cells,^{15–17} since they allow free exchange of proteins, metabolites, nutrients, oxygen, and anticancer drugs when dispersed in the cell culture medium.^{18–20} Typically, the droplet-microfluidic technique is a powerful tool to fabricate mono-disperse hydrogel microparticles using shape and size-controllable droplets as templates.^{21–26} Microscale hydrogel scaffolds made of PEG, PNIPAM, or gelatin methacrylate (Gel-MA) have been produced by using UV- or thermo-induced cross-linking, in which potential harmful radicals may damage cells.^{27,28} On the contrast, alginate is a highly hydrated ion-induced cross-linking polymer network that exhibits excellent biocompatibility for cell encapsulation.^{29,30} The cross-linked alginate networks are commonly used to support cell culture; however, there is a problem in controlling their permeability and degradation for cell culture and release.^{31,32} To address this issue for cell culture, the properties of the alginate hydrogel can be modified by chemical derivatives for controlling cell interaction,³³ physical stiffness,³⁴ and covalent cross-linking.³⁵ Moreover, the alginate degradation has become a major concern because of its inherent non-degradation. In typical, periodate oxidation was developed to control the degradation of alginate scaffolds;³⁶ however, this method had potential hurt to the cells. Degradation of the alginate hydrogel was also controlled by adjusting the molecular weight distribution of alginate,³⁷ but the degradation rate cannot be finely controlled.

In this paper, we report a convenient method to achieve flexible control of gelation, permeability, and degradation of droplet-templated hydrogel scaffolds for cellular encapsulation and release in microfluidics. We synthesize a functional diblock copolymer, alginate-conjugated PNIPAM (Alg-co-PNIPAM), and subsequently fabricate it into lots of micrometer-sized hydrogel microparticles by using water-in-oil (w/o) emulsions as templates in a designated microfluidic flow-focusing device. In a typical experiment, we make a non-harmful cross-linking of the Alg-co-PNIPAM scaffold through quickly triggered release of Ca^{2+} from the Calcium-EDTA complex by diffusing H^+ into the premicrogel droplets. The synthetic microparticles enable to form many nano-aggregates in non-uniform hydrogel networks through controlling temperature at low critical solution temperature (LCST), which could modify the permeability of hydrogel scaffolds. We further demonstrate the successful fabrication of mono-disperse cell-laden microgel particles at such a mild condition with high cell viabilities. Most importantly, remotely controlled release of microencapsulated cells from the smart microparticles is achieved via cell-compatible hydrogel degradation process through competitive affinity of Ca^{2+} between alginate units and EDTA^{2-} . Thus, this bifunctional copolymer for flexible control of cellular microencapsulation offers an excellent alternative for fabrication of non-harmful hydrogel scaffolds, which could be useful in cell delivery, tissue engineering, drug screening, and other basic biomedical researches.

EXPERIMENTAL

Synthesis of alginate-conjugated PNIPAM: Alginate (MVG alginate, medium viscosity > 200 mPa s, PRONOVA, MA, USA) was chemically conjugated with NH_2 -PNIPAM (Poly (N-isopropylacrylamide), amine terminated average; Mn 5500) based on the typical EDC-NHS reaction between its carboxylic acid group and $-\text{NH}_2$. Briefly, 500 mg of MVG alginate was dissolved into 5.0 ml PBS buffer overnight. After that, 500 mg NH_2 -PNIPAM, 800 mg N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC, Sigma, USA), and 40 mg N-hydroxysuccinimide (NHS, sigma, USA) were successively added into the alginate solution under a stirring condition at room temperature. After 24 h, the product was transferred into dialysis tubing (MwCO: 3500). In a 4 l bucket, the dialyzing purification was performed in deionized water for 5 days by changing the solution 2–3 times per day. Finally, the product was further concentrated by rotary evaporation. A concentration of 0.8% Alg-co-PNIPAM (w/v) was obtained for further experiments by storing at 4 °C condition.

Microfluidic device fabrication: The microfluidic chip for droplet generation was fabricated by traditional standard soft lithography.^{38,39} In a typical experiment, a mask was designed by computer-aided design (CAD 2012). The SU-8 2050 photoresist (MicroChem Co., MA, USA)

was spin-coated onto a silicon wafer with a height of 100 μm . After exposure by UV light under the mask, the SU-8 coated master mold was developed by propylene glycol methyl ether acetate (PGMEA). Further, a mixture of poly(dimethylsiloxane) (PDMS) with the initiator agent (Sylgard 184 elastomer kit, Dow Corning, w/w = 10:1) was poured onto the master mold. The PDMS prepolymer was degassed, and then solidified for 2 h at 75 °C. After that, the PDMS replica was peeled off, punched to make holes, and bonded onto a glass slides by air plasma treatment. Finally, we hydrophobically treat the microchannels with Aquapel[®] (PPG, Pittsburgh, PA, USA).

Cell culture and fluorescent staining: Human hepatocellular carcinoma cell (HepG2) line was obtained from Brigham and Women's Hospital at Harvard Medical School. The HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin, and penicillin. The HepG2 cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. The HepG2 cells were passaged every 3 days. For cell encapsulation experiments, the HepG2 cells were digested by 1% Trypsin and resuspended in DMEM medium with a density of 5.0×10^7 cells/ml. After the HepG2 cells were encapsulated in the microgels, they were transferred into the cell culture medium containing 7.5 mM CaCl₂ for further cross-linking. The cell-laden microparticles were cultured by culture dishes at 37 °C in 5% CO₂. The HepG2 cells in the microparticles were stained by LIVE/DEAD[®] Viability/Cytotoxicity Kit (Life Technologies, Inc., USA) for cell viability assays. A confocal microscope (Leica TCS SP5) was used for fluorescent analysis of cells.

Fabrication of cell-laden microparticles: The droplet-templated cell-laden microparticles were produced by the designated microfluidic flow-focusing device. The generation of droplet emulsions was recorded using a IX2-SLP phase inverted microscope installed with a fast-camera (SONY XCDV60, Japan). The innermost aqueous phase was HepG2 cells contained culture medium. The middle aqueous phase was a solution containing 100 mM Ca-EDTA, 1.0% Alg-co-PNiPAM, and 1.0% MVG-alginate (medium viscosity > 200 mPa s, PRONOVA, MA, USA). The fluorinated surfactant (Perfluorinated polyethers-polyethyleneglycol, PFPE-PEG) was purchased from RainDance Technologies (MA, USA). The inner oil phase was HFE-7500 containing 1.0% surfactant. The outer oil phase was HFE-7500 containing 1.0% surfactant and 0.15% acetic acid. All the solutions were transferred into a plastic syringe (Becton, Dickinson and Company). The syringes were connected with a microfluidic device by several well-matched PE tubing. Two Harvard Precision pumps were used to control the flow rate. The droplets were generated by the microfluidic flow-focusing device and then collected into HFE-7500 oil without acetic acid. Finally, the oil phase was removed by cell strainer (40 μm , BD Falcon[™], USA) and the cell-laden microparticles were transferred into cell culture medium containing 7.5 mM CaCl₂.

Dynamic light scattering (DLS) was applied to analyze hydrodynamic size distributions of nano-segregates in the Alg-co-PNiPAM hydrogel. The DLS instrumentation was operated via a Malvern Zetasizer NanoS instrument in the temperature range from 24 °C to 40 °C. DLS measurements for each temperature point were taken after equilibrating for 5.0 min. All size distributions are averages of three measurements.

RESULTS AND DISCUSSION

Synthesis of Alg-co-PNiPAM diblock copolymer

Hydrogel is a highly hydrated, cross-linking polymer that shows very important biological applications in cell researches.⁴⁰ To achieve a flexible control of cell microencapsulation in a hydrogel scaffold, we designed and synthesized a functional diblock copolymer, Alg-co-PNiPAM, and used it as cell-compatible hydrogel matrixes for cell encapsulation. The synthetic strategy of Alg-co-PNiPAM diblock copolymer is shown in Fig. 1(a), in which carboxylic groups of alginate chains are chemically conjugated with amino-terminated PNIPAM via a typical EDC-NHS reaction. We further conjugate alginate chains with amino-terminated fluorescein to demonstrate this reaction (see [supplementary material](#), Figs. S1 and S2). Subsequently, we

use the microfluidic technique to produce uniform micrometer-sized premicrogels containing Alg-co-PNiPAM copolymer and Ca-EDTA solution from droplet templates (Fig. 1(b)). In particular, in this hydrogel scaffold, the alginate chain can form a porous stable network cross-linked by Ca^{2+} , once H^+ diffuses from the surround oil phase into the droplet by triggering the release of Ca^{2+} from the Ca-EDTA complex (see [supplementary material](#), Fig. S3(a)). Thus, reversible degradation of hydrogel scaffolds can be achieved via competitive affinity of Ca^{2+} by EDTA^{2-} anion from Calcium-Alginate networks; the balance is expressed as

thereby the hydrogel is immediately dissolved when the Na_2EDTA solution ($\text{pH} = 7.0$) is introduced (see [supplementary material](#), Fig. S3(b)). This method offers a very mild condition to control the gelation and degradation of hydrogel scaffolds for non-harmful cross-linking and degradation through ion-mediated affinity.

Design of microfluidic flow-focusing device and microgel fabrication

We used a droplet-based microfluidic technique to fabricate mono-disperse hydrogel micro-particles. In a typical experiment, we designed a microfluidic flow-focusing device for *in-situ* gelation of hydrogel microparticles on-chip. Two aqueous phases, cell culture medium and Alg-co-PNiPAM solution (1.5%, w/w) containing 100 mM Ca-EDTA ($\text{pH} = 7.0$), were respectively directed into the inner channels by syringes, as shown in Fig. 2(a). Note that the inner culture medium phase will be employed to flow mammalian cells for controlling cell microencapsulation. Under the fluid shear stress of the immiscible oil phase (HFE-7500 with 1.0% surfactant), the pre-microgel droplets were first formed in the microchannels (Fig. 2(b)). Subsequently, we introduce another oil phase containing 0.15% acetic acid into the second microchannels, where the H^+ in the oil phase diffuses into the premicrogel droplets, cross-linking the hydrogel networks by quickly triggering the release of Ca^{2+} in the Calcium-Alginate complex (Figs. 2(c) and 2(d)). These two oil phases are amenable to the formation of droplets and microgels, respectively. Using these water-in-oil (w/o) emulsions as templates, we fabricate lots of monodisperse hydrogel microparticles (Fig. 2(e)) and then collect them into the tubes with non-acid contained oil. Thus, we are able to produce uniform hydrogel scaffolds with a well-controlled structure with a diameter of $143.2 \pm 6.3 \mu\text{m}$ (Figs. 2(f) and 2(g)).

FIG. 1. Schematic diagram of the synthetic route to Alg-co-PNiPAM polymer by conjugating alginate with PNIPAM via the EDC-NHS reaction process. The red and green lines represent the blocks of alginate and PNIPAM, respectively. (b) Illustration of the generation of hydrogel scaffolds via droplet templates in microfluidics. A microscale droplet containing Alg-co-PNiPAM and Ca-EDTA mixtures was generated in a microfluidic flow-focusing device, and gelated by H^+ diffusion from oil phase. The gelation of hydrogel scaffolds can be released by competitive affinity of Ca^{2+} by EDTA^{2-} .

FIG. 2. Microfluidic fabrication of cell-laden hydrogel microparticles using droplets as templates. (a) and (b) Schematic illustration of the microfluidic flow-focusing device. It contains culture medium as an inner aqueous phase, Alg-co-PNiPAM and Ca-EDTA solution as the second aqueous phase, HFE-7500 and surfactant as the break oil phase, and a cross-linking phase containing acetic acid. (c) and (d) Formation of Alg-co-PNiPAM microgels via triggered release of Ca^{2+} on chip. Acetic acid in oil phase diffuses into the droplets. (e) and (f) Images of stable droplets in the flow microchannel, and collected in the oil container. (g) Size distribution of the monodisperse gel-in-oil droplets. 100 droplets were counted with an average diameter of $143.2 \pm 6.3 \mu\text{m}$.

Physicochemical control of hydrogel scaffolds

The fabricated hydrogel microparticles are endowed with an additional function of physicochemical control of the permeability in hydrogel scaffolds because of the introduced PNIPAM chains in the synthetic Alg-co-PNiPAM diblock copolymer. A microgel with non-uniform hydrogel networks can be remotely controlled via the thermo-responsive property of this diblock copolymer (Fig. 3(a)), when the PNIPAM chains become nano-aggregates in the hydrogel microparticles by raising the temperature above LCST (Fig. 3(b); see [supplementary material](#), Figs. S3(c) and S3(d)). We characterize the statistic distribution of nano-sized aggregates in the Alg-co-PNiPAM hydrogel (1.5%, m/m) by the measurement of dynamic light scattering (DLS). As shown in Fig. 3(c), an average hydrodynamic diameter of polymer nano-aggregates in the hydrogel microcapsules is below 50 nm at low temperature, while the nano-aggregates of Alg-co-PNiPAM polymers quickly increase to $\sim 500\text{nm}$ at a temperature of above 32°C (LCST). To clearly observe this change, we further use Rhodamine-B, a conventional red fluorescent dye, to stain the hydrogel networks in the hydrogel microparticles. Under a fluorescence confocal microscope, there are different morphologies of nano-aggregates in the hydrogel microparticles by switching the temperature from 30°C to 40°C , as shown in the inset of Fig. 3(c). The statistic analysis of size distribution by intensity also clearly indicates this change under the temperature control (see [supplementary material](#), Fig. S4). These results suggest that

FIG. 3. Physicochemical characterization of the functional hydrogel microparticles. (a) and (b) Illustration of the thermo-responsive characteristic of microparticles via temperature change. The PNiPAM blocks become aggregates above the LCST. (c) Dynamic light scattering (DLS) test of Alg-co-PNiPAM solution in the temperature range from 24 °C to 40 °C. Inserted images were optical micrographs of rhodamine-B containing Alg-co-PNiPAM microgels at $T < LCST$ and $T > LCST$. LCST is 32 °C for the Alg-co-PNiPAM hydrogel.

it is possible to achieve a physicochemical control on the permeability of hydrogel scaffolds, since the nano-aggregates can greatly change the porosity density of hydrogel scaffolds, forming many non-uniform porous rigid networks in the microparticles. It should be noted that the standard temperature for cell culture condition is 37 °C, which is located above the LCST (32 °C); thereby the enlarged permeability of the hydrogel microparticles will allow a better permeability of large substances such as enzymes, antibodies, and leucocytes for tissue engineering. Thus, the synthetic microparticles enable us to achieve flexible control of the permeability of hydrogel scaffolds for cell microencapsulation.

Mammalian cell encapsulation and viability assays

Encouraged by the excellent properties of the fabricated hydrogel microparticles, we try to achieve a flexible control of cell microencapsulation in the hydrogel scaffolds. In this work, we use HepG2 cells as a model of mammalian cells. We inject the HepG2 cells (5.0×10^7 cells ml^{-1}) into the innermost channels, producing a large number of cell-laden microgels by using the designed microfluidic flow-focusing device (see [supplementary material](#), Fig. S5). In this case, the HepG2 cells are on-chip locked inside the hydrogel microparticles, and the cell-laden microgels are quickly collected into a pure oil phase for diluting the acetic acid and eliminating potential damages to the HepG2 cells (Fig. 4(a)); thereafter, we remove most of the oils and transfer them into cell culture medium containing 7.5 mM CaCl_2 for further gelation of Alg-co-PNiPAM hydrogel scaffolds (Fig. 4(b)). Note that the residual oils evaporate quickly at 37 °C, and thus, all the cell-laden microparticles settle into cell culture medium for long-term cell culture at 37 °C and 5% CO_2 . All the cell-laden microgels are mono-disperse and stable by maintaining their spherical shape even for several days of culture. Subsequently, the cell viability in the hydrogel microparticles is examined by using a cell live/dead assay kit (Calcein AM/EthD-1): Calcein AM is a green fluorescent dye for staining live cells, whereas EthD-1 is a red fluorescent dye for staining dead cells. As shown in Figs. 4(c) and 4(d), the microencapsulated cells have a high viability surviving in the hydrogel scaffolds. At day 3, the cell viability has a slight decrease to 93% because of the

FIG. 4. Cell microencapsulation in the hydrogel microparticles. (a) and (b) Highly monodisperse, stable cell-laden microgels in oil and cell culture medium, respectively. (c) Viability of microencapsulated cells in the hydrogel microparticles after 24 h culture. HepG2 cells were stained by the live/dead kit (Calcein AM/EthD-1). (d) Percentage viability of microencapsulated cells in the hydrogel microparticles for 1–3 days.

cell growth and formation of dense cell aggregates that lead to compete nutrients or oxygen each other, and thus cause cell death. These results demonstrate the cell-compatibility of the synthetic hydrogel microparticles for their feasible applications in cell delivery and tissue engineering.

Remote control of releasing microencapsulated cells

As the benefit of the synthetic diblock copolymer, we realize that the hydrogel microparticles can not only enable precisely controlling cellular microencapsulation in a more permeable scaffold but also allow remotely controlling the release of microencapsulated cells, inspired by the competitive affinity of Ca^{2+} with the alginate unit. In this regard, we find an interesting approach to achieve the degradation of the hydrogel scaffolds by introducing Na_2EDTA solution as competitive binding reagents. In a typical experiment, the HepG2 cells are encapsulated in separated microgel scaffolds, maintaining their cell survives in the cell culture medium, as shown in Figs. 5(a) and 5(d). We clearly observe that the hydrogel scaffolds are immediately dissolved when a drop of Na_2EDTA solution (100 mM, $\sim 50 \mu\text{l}$, $\text{pH} = 7.0$) is introduced (Figs. 5(b) and 5(e)); the remote control of triggered degradation of such hydrogel scaffolds has been successfully achieved because of strong binding balance to Ca-EDTA , as described in Fig. 1(b). As such, we are able to release the microencapsulated cells from the hydrogel microparticles and disperse them into the cell culture medium by a slight shaking (Figs. 5(c) and 5(f)). Here, all the cells are stained with Calcein AM/EthD-1 to monitor their viability under a fluorescence confocal microscope. It is important to note that the proposed strategy for ion-induced gelation and degradation of the hydrogel scaffolds is of great significance in biomaterial science. This result also suggests that we have successfully developed an efficient,

FIG. 5. Controlled release of microencapsulated cells from the hydrogel microparticles. (a) and (d) Bright field and fluorescent images of encapsulated cells in the microgels. (b) and (e) Degradation of the hydrogel scaffold by introducing Na_2EDTA solution for competitive affinity of Ca^{2+} . (c) and (f) Triggered release of microencapsulated cells from the hydrogel microparticles and free dispersion. The fluorescence was the stained cells with the Calcein AM/EthD-1 kit.

convenient method to achieve flexible control of cellular microencapsulation in a droplet-templated hydrogel scaffold at a very mild condition by designing the Alg-co-PNIPAM diblock copolymer.

CONCLUSIONS

We use droplet-based microfluidics to fabricate lots of mono-disperse, micrometer-sized hydrogel scaffolds for achieving flexible control of cellular encapsulation, permeability, and release. As benefits of the excellent property of the synthetic diblock copolymer, we have successfully developed an approach for controlling cellular encapsulation and release upon the gelation, permeability, and degradation of the droplet-templated hydrogel scaffolds via the ion-exchange and temperature. This approach allows us to maintain the cell-laden microgels with their spherical shapes, and an excellent cellular microenvironment for cell survives at a mild condition, which we have demonstrated the cell-compatibility of hydrogel scaffolds through the radical-free, non-harmful cross-linking with their high cell viabilities. Therefore, we envision that the developed strategy for cell microencapsulation would have many exciting biomedical applications in drug screening, cellular delivery, and tissue engineering in the future.^{41–43}

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for chemical synthesis and physical characterization of diblock copolymer. Cell encapsulation in droplet-based microfluidic device is shown.

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