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Production of hyaluronic-acid-based cell-enclosing microparticles and microcapsules via enzymatic reaction using a microfluidic system

Mehdi Khanmohammadi, Shinji Sakai, Tomoaki Ashida, Masahito Taya
Division of Chemical Engineering, Department of Materials Science and Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka Osaka Japan
Correspondence to: S. Sakai (E-mail: sakai@cheng.es.osaka-u.ac.jp) and M. Taya (E-mail: taya@cheng.es.osaka-u.ac.jp)

ABSTRACT: This article describes the preparation of cell-enclosing hyaluronic acid (HA) microparticles with solid core and microcapsules with liquid core through cell-friendly horseradish peroxidase (HRP)-catalyzed hydrogelation. The spherical vehicles were made from HA derivative possessing phenolic hydroxyl moieties (HA-Ph) cross-linkable through the enzymatic reaction by extruding cell-suspending HA-Ph aqueous solution containing HRP from a needle of 180 μm in inner diameter into the ambient coaxial flow of liquid paraffin containing H_2O_2 in a microtubule of 600 μm in diameter. By altering the flow rate of liquid paraffin, the diameters of gelatin and HA-Ph microparticles were varied in the range of 120–220 μm and 100–300 μm, respectively. The viability of the enclosed human hepatoma HepG2 cells in the HA-Ph microparticles of 180 μm in diameter was 94.2 ± 2.3%. The growth of the enclosed HepG2 cells was enhanced by decreasing the HRP concentration. The microcapsules of 200 μm in diameter were obtained by extruding HA-Ph aqueous solution containing thermally liquefiable cell-enclosing gelatin microparticles of 150 μm in diameter using the same microfluidic system. The enclosed cells grew and filled the cavity within 10 days. Spherical tissues covered with a heterogeneous cell layer were obtained by degrading the microcapsule membrane using hyaluronidase after covering the surface with a heterogeneous cell layer. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43107.

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INTRODUCTION

Hyaluronic acid (HA) is an anionic polysaccharide and inherently present in the human body as a key component of the extracellular matrix.[1] It is a nonadhesive and hydrophilic, negatively charged polymer composed of repeating disaccharide units of β-1,4-linked glucuronic acid and β-1,3-linked *N*-acetyl-D-glucosamine connected by glycosidic bonds. Due to superior biocompatibility and cytocompatibility, HA has been used as a useful material in medical and pharmaceutical applications.[2–4] For instance, HA has been injected into the joints for improving lubrication and under the skin in cosmetic surgery.[2,5]

In this study, we aimed to develop HA-based mammalian cell-enclosing microparticles and microcapsules. Mammalian cell-enclosing spherical vehicles composed of hydrogel have been studied for more than 40 years for applications to transplantation of cells,[6,7] production of biological macromolecules,[8] and tissue engineering.[9,10] Due to the solubility in water, it is impossible to obtain cell-enclosing spherical vehicles from unmodified HA alone. A possible approach is fabrication of polyelectrolyte membranes with cationic polymers on spherical templates.[11] Another approach is the use of chemically modified derivatives. HA-based hydrogel microparticles have been prepared from photo-crosslinkable derivatives by using alginate as a temporal spherical mold.[12,13] In this study, we attempted to use a derivative of HA possessing phenolic hydroxyl moieties (HA-Ph) cross-linkable through horseradish peroxidase (HRP)-catalyzed oxidative reaction. In the enzymatic hydrogelation system, the enzyme catalyzes the cross-linking of Ph moieties using H_2O_2 as an electron donor [Figure 1(a)].[7,10] HA-Ph has firstly been developed by Kurisawa *et al.*[14] The biocompatibility and attractiveness of HA-Ph hydrogels have been revealed both *in vitro* and *in vivo* studies as materials of carriers for drug deliveries and tissue engineering.[8,11] However, as long as we know, no attempts have been performed for the preparation of cell-enclosing spherical HA vehicles obtained through the cross-linking of HA and HA-based molecules.

For obtaining cell-enclosing HA-Ph microparticles and microcapsules, we used a microfluidic coaxial double-orifice spinneret which has been used for obtaining spherical[7,10,15] and tubular[16] vehicles from alginate[7] and cellulose[15] derivatives. The HA-Ph microcapsule preparation technique involves a process of

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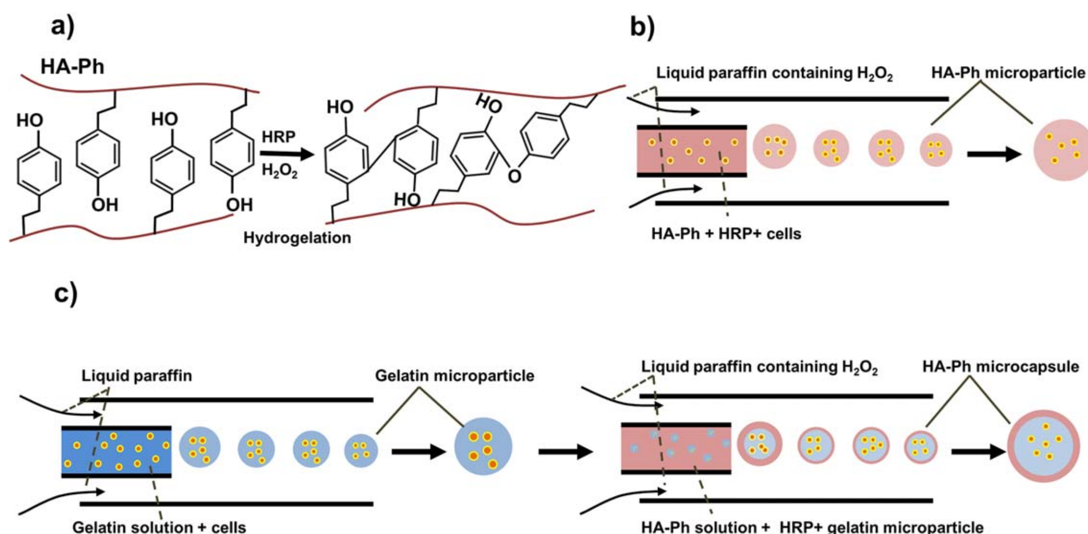

Figure 1. Schematic illustrations of (a) conjugated hydrogel formation through HRP-catalyzed cross-links between Ph moieties in HA-Ph for fabrication of microcapsules and microparticles, (b) production process of HA-Ph microparticles enclosing HepG2 cells, and (c) production process of gelatin microparticles enclosing HepG2 cells, followed by encapsulating in HA-Ph microcapsules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

droplet breakup in a coflowing water-immiscible fluid via formation of a thin jet of an aqueous polymer solution (coflowing method).[17] In this study, HA-Ph aqueous solution containing HRP was extruded from an inner needle into a surrounding flow of liquid paraffin containing H_2O_2. The extruded HA-Ph aqueous solution forms droplets and then hydrogelated microparticles through the enzymatic reaction by consuming H_2O_2 supplied from ambient liquid paraffin [Figure 1(b)]. HA-Ph microcapsules are obtained by extruding HA-Ph solution containing HRP and gelatin microparticles [Figure 1(c)] and subsequent thermal liquefaction of gelatin microparticles by incubating at 37°C. In addition, the growth profiles of the enclosed cells and the possibility of fabricating spherical tissues covered with a heterogeneous cell layer by degrading the HA-Ph microcapsule membrane using hyalurodinase were investigated to reveal the potential of the resultant cell-enclosing vehicles in tissue engineering applications.

MATERIALS AND METHODS

Materials

Sodium HA (MW 1.79×10^6 Da) was obtained from the JNC Corp. (Tokyo, Japan). *N*-Hydroxysuccinimide (NHS), HRP (210 units/mg), lecithin from soybean and H_2O_2 aqueous solution (30% (w/w)) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Tyramine hydrochloride and liquid paraffin were products from Kanto Chemical (Tokyo, Japan). Water-soluble carbodiimide hydrochloride (WSCD) and 2-morpholinoethane sulfonic acid (MES) were obtained from Peptide Institute (Osaka, Japan) and Dojindo Molecular Technologies (Kumamoto, Japan), respectively. Gelatin (Type A from porcine, 300 Bloom) and hyaluronidase from the sheep testes ($300 \geq$ units/mg solid) were purchased from Sigma (St. Luis. MO, USA). A human hepatoma cell line, HepG2 cells, and human cervical cancer cell line, HeLa cells, were obtained from Riken Cell Bank (Ibaragi, Japan). These cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS), 75 μg/mL penicillin and 50 μg/mL streptomycin under a humidified atmosphere of 5% CO_2/95% air at 37°C.

Synthesis of HA-Ph and Gelatin-Ph

HA-Ph and gelatin-Ph were synthesized based on the reported methods by conjugating HA and gelatin with tyramine using WSCD and NHS.[14,18] Briefly, HA or gelatin was dissolved in 50 mM MES buffer (pH 6.0) at 10 or 20 g/L, respectively. To this solution, WSCD, NHS, and tyramine hydrochloride were added at 4.7, 2.9, and 5.0 g/L for HA-Ph synthesis and 7.4, 2.3, and 10 g/L for gelatin-Ph synthesis, respectively. The mixture was stirred by a magnetic bar for 20 h at room temperature. Remaining chemicals in the resultant solution were removed by precipitation with 90% (v/v) ethanol aqueous solution for HA-Ph synthesis and dialysis against distilled water for gelatin-Ph synthesis. The phenol moieties in HA-Ph and gelatin-Ph were 1.6×10^{-4} Ph moieties/g HA-Ph and 1.1×10^{-4} Ph moieties/g gelatin-Ph.

Production of Microparticles and Microcapsules

Cell-enclosing HA-Ph microparticles were prepared using a microfluidic co-axial double-orifice spinneret.[10] Briefly, phosphate-buffered saline (PBS, pH 7.4) containing 0.75% (w/v) HA-Ph, 1×10^7 cells/mL HepG2 cells, and 30, 60, or 100 units/mL HRP was made to flow from an inner tubule of 180 μm inner diameter at 4.9 cm/s and liquid paraffin containing 3% (w/w) lecithin and H_2O_2 was extruded through an outer tube with diameter of 600 μm. The HA-Ph microdroplets suspended in the liquid paraffin became gelled during the flow and were collected. After 10 min of standing, the resultant cell-enclosing HA-Ph microparticles were finally transferred into medium via centrifugation.

Cell-enclosing HA-Ph microcapsules were prepared using the above-mentioned microfluidic system. Cell-enclosing gelatin microparticles were used for templating cavities in the capsules.

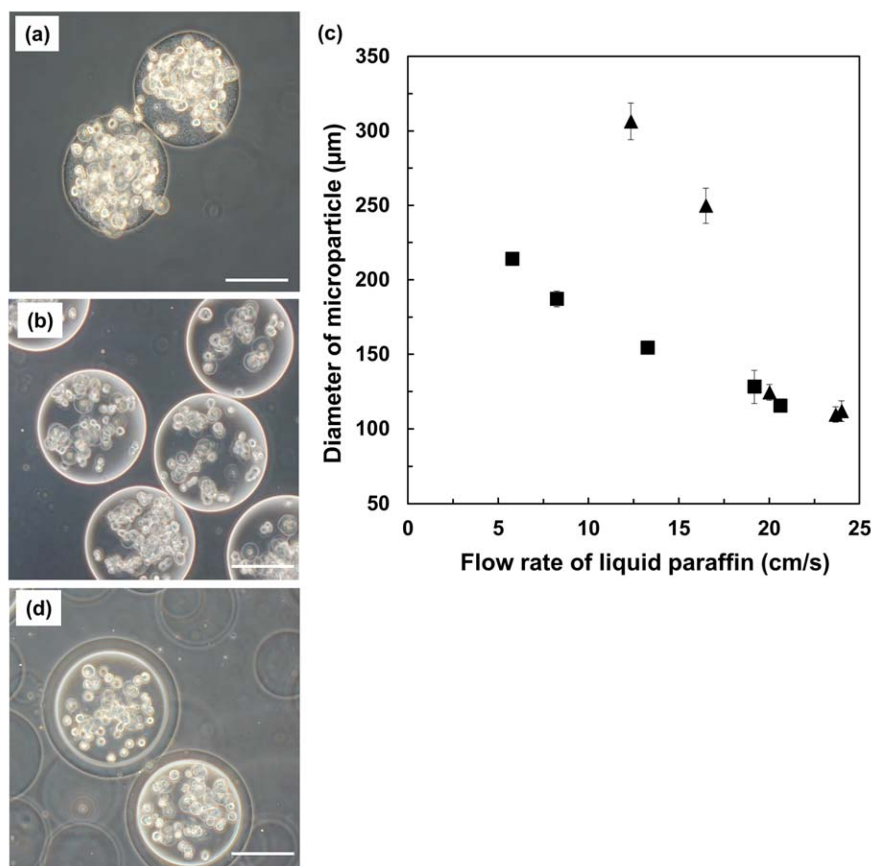

Figure 2. Typical microphotographs of (a) HA-Ph and (b) gelatin microparticles, and (d) HA-Ph microcapsules. (c) Dependence of diameters of gelatin microparticles (■) and HA-Ph microparticles (▲) on flow rate of liquid paraffin containing H_2O_2. The flow rates of gelatin and HA-Ph solutions were fixed at 4.5 mL/h in the experiments for testing the effect of flow rate of liquid paraffin. Horizontal bars: 100 μm and vertical bars: SD ($n = 100$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The gelatin microparticles were obtained by extruding PBS containing 7.5% (w/v) nonmodified gelatin and 1×10^7 cells/mL HepG2 cells at 37°C from an inner tube at 4.9 cm/s into a co-axial flow of liquid paraffin. The resultant emulsion system was cooled in an ice bath for obtaining gelatin microparticles. After collecting the microparticles in PBS, they were suspended at a ratio of 1:10 (w/v) in PBS containing 0.75% (w/v) HA-Ph and 100 units/mL HRP. Then the solution was extruded from an inner needle into a co-axial flow of liquid paraffin containing 3% (w/w) lecithin and H_2O_2 at 25°C. After 10 min of standing, the resultant HA-Ph microparticles containing cell-enclosing gelatin microparticles were transferred into medium via centrifugation. The HA-Ph microcapsules containing spherical cavities were obtained by incubating the HA-Ph microparticles at 37°C for thermal gel-to-sol transition of un-modified gelatin hydrogel.[19]

Effect of Encapsulation on Cells

At 1 h after encapsulation, the enclosed cells were harvested from HA-Ph microparticles and microcapsules by soaking in medium containing 0.15 mg/mL hyaluronidase for 30 min. Then the viability of the harvested cells was determined by trypan blue exclusion dye assay. In addition, the harvested HepG2 cells were seeded at 1×10^4 cells/cm^2 on a 48-well tissue culture dish with DMEM supplemented 10% (v/v) FBS for evaluating the possible occurrence of adverse effects requiring time to induce cell death and growth.

Mechanical Properties

To measure the mechanical property of HA-Ph hydrogel, the synthesized polymer was dissolved at 0.75% (w/v) in PBS at 37°C and then was mixed with HRP at 30, 60, and 100 units/mL in the presence of 1 mM H_2O_2 in a cylinder module. The compression–repulsion force profiles were measured using the cylindrical hydrogels with dimension of approximately 12 mm diameter and 4 mm thickness. The gels were placed in a Table-Top Material Tester (EZ Test 500N, Shimadzu, Kyoto, Japan) installed with a flat probe of 8 mm in diameter and compressed at a crosshead speed of 2.0 mm/min. The load was applied until 30% compression was achieved.

Fabrication of Spherical Tissues

After 10 days of incubation of cell-enclosing microcapsules for yielding the cavities filled with the cells, the microcapsules were soaked in medium containing 0.15 mg/mL hyaluronidase for obtaining the naked HepG2 spherical tissues. For preparing the spherical tissues covered with different cells, the microcapsules were soaked in PBS containing 0.1% (w/v) gelatin-Ph, 0.2 units/mL HRP, and 0.3 mM H_2O_2 for 1 min. Through this process,

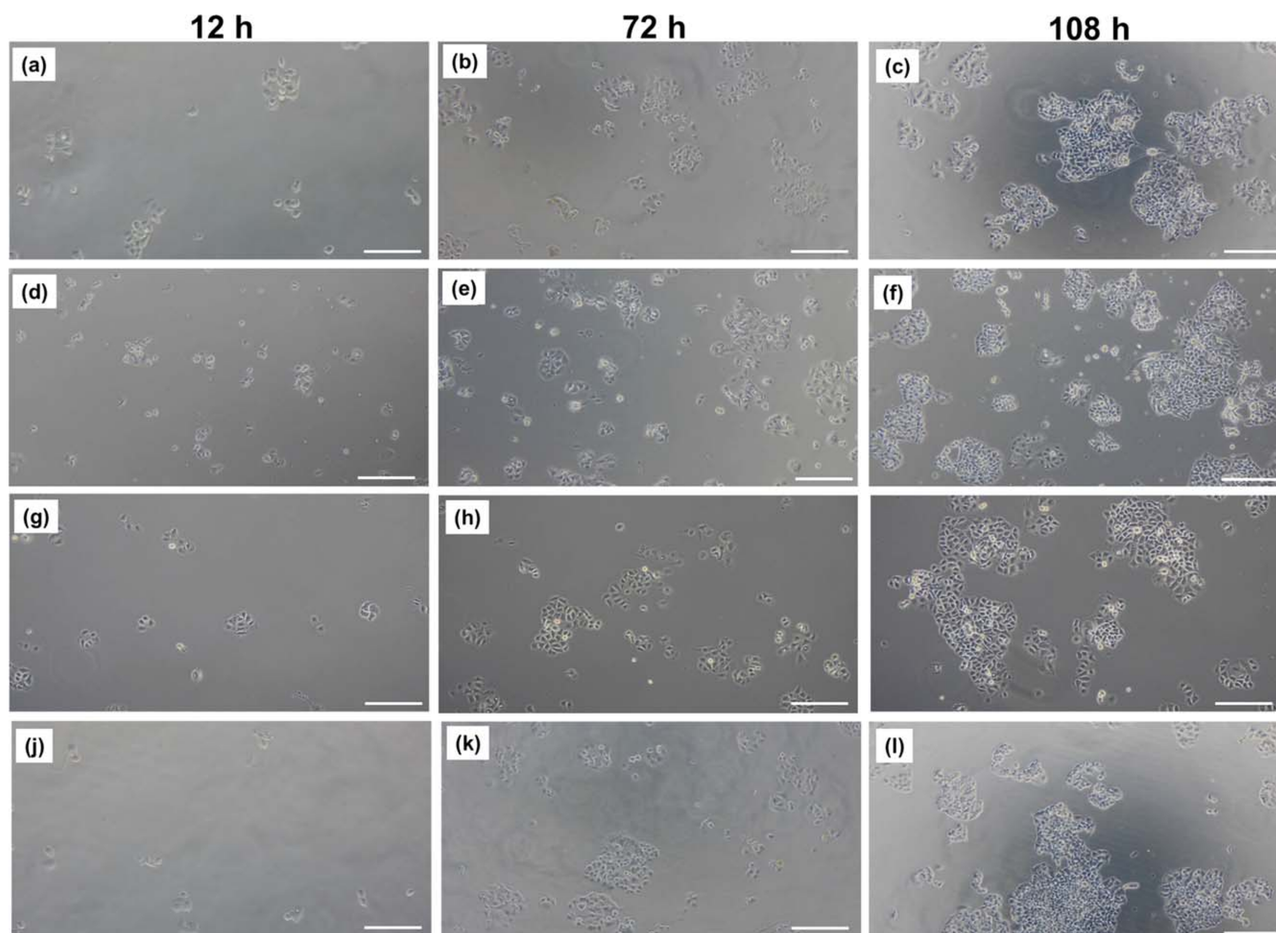

Figure 3. Microphotographs of HepG2 cells cultured on culture dishes. (a–c) Control (conventional subculture). Cells harvested from (d–f) gelatin microparticles, (g–i) HA-Ph microparticles and (j–l) HA-Ph microcapsules by degradation of HA-Ph membrane using hyalurodinase. Horizontal bars: 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the surfaces of the HA-Ph microcapsules were coated with gelatin-Ph. Then, the microcapsules were suspended in a medium containing 2×10^5 cells/mL HeLa cells on a cell culture dish treated with 1% (w/v) agarose gel. After 1 day of incubation to allow attachment and growth of HeLa cells on the microcapsules, the microcapsules were soaked in the medium containing 0.15 mg/mL hyaluronidase. The HepG2 cells and HeLa cells stained with Cell Tracker Green (Life Technologies, NY, USA) and Cell Tracker Orange (Life Technologies), respectively, were used to facilitate visual observation.

RESULTS AND DISCUSSION

Hydrogels of HA-Ph, a derivative of HA well-known as a biocompatible material, obtained through HRP-catalyzed reaction have been investigated for various biomedical applications such as drug delivery.[14,20,21] To date, the superior biocompatibility of the HA hydrogels has been revealed in the studies using animals.[20–22] These indicate that HA-Ph hydrogel is an attractive material as cell-enclosing microparticles and microcapsules which can be used for cell delivery *in vivo* and tissue engineering *in vitro*. In the encapsulation of cells, the HA-Ph hydrogel has advantages in comparison with photo-crosslinkable HA and polyelectrolyte HA-based hydrogels.[11,23] The photo-crosslinkable HA hydrogel is produced by exposure of UV light which can be cytotoxic and preclude encapsulation of vital cells.[23] Furthermore, polyelectrolyte HA-based hydrogel exhibits inherent structural instability due to weak electrostatic interactions.[11,23] The motivation of this study was to investigate the possibility of preparing the cell-enclosing spherical vehicles from HA-Ph, the behavior of enclosed cells in HA-Ph spherical vehicles, and the applicability of the vehicles for spherical tissue fabrications.

Preparation of Gelatin and HA-Ph Hydrogel Microparticles

Firstly, we studied the relation between flow rate of liquid paraffin and diameter of resultant HA-Ph microparticles. As we expected, HA-Ph microparticles could be obtained using the microfluidic system [Figure 2(a)] which has been used for preparing microparticles from other polymeric aqueous solutions including gelatin microparticles [Figure 2(b)].[17,24,25] The diameters of spherical vehicles were measured from images obtained using optical microscope. The diameter of HA-Ph microparticles decreased with increasing flow rate of liquid paraffin [Figure 2(c)]. This tendency was in agreement with the previous results obtained for the microparticles prepared from other polymeric aqueous solutions[17,24,25] and gelatin aqueous solution

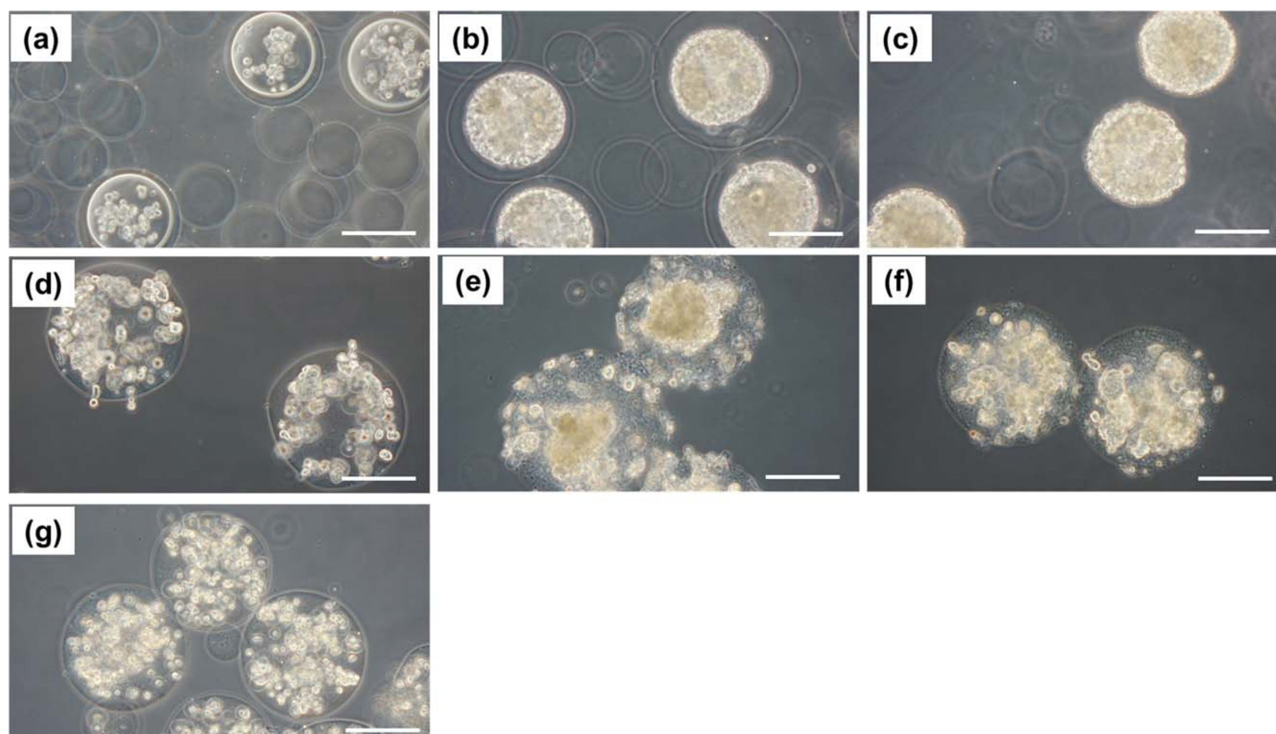

Figure 4. Microphotographs of HepG2 cells grown in HA-Ph (a–c) microcapsules and (d–g) microparticles. (a) At 4 h of encapsulation, (b) at 8 days of encapsulation, and (c) after degradation of HA-Ph membrane by hyalurodinase at 8 days of encapsulation. (d) At 4 h of enclosing in microparticles, and (e–g) at 11 days of enclosing in microparticles prepared at 30, 60, and 100 units/mL HRP, respectively. Horizontal bars: 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[Figure 2(c)]. In detail, the diameter of HA-Ph microparticle decreased from 300 to 100 μm with increasing flow rate of liquid paraffin from 12.3 to 24.1 cm/s. The difference in diameters of HA-Ph microparticles and gelatin microparticles would be induced by the difference in the viscosities of HA-Ph and gelatin solutions. It is known that smaller droplets formed from a higher viscous solution.[26] In fact, the viscosities of 0.75% (w/v) HA-Ph solution at 25°C and 7.5% (w/v) gelatin solution at 37°C were 264 and 14 mPa s, respectively. The narrow size distributions of the resultant HA-Ph and gelatin microparticles were confirmed from the coefficients of variance (defined as a standard deviation divided by average diameter of microparticles) of less than 6% under all the conditions. In addition, HA-Ph microcapsules also could be obtained from HA-Ph solution containing gelatin microparticles as a template of cavities [Figure 2(d)], likewise in the case of the microcapsules obtained from an alginate derivative possessing Ph moieties (Alg-Ph).[27] In the production of HA-Ph microcapsules, the gelatin microparticles were suspended at a ratio of 1:10 (w/v) in a large volume of HA-Ph precursor solution. This condition could assure the entrapment of single gelatin microparticle in each HA-Ph emulsion droplet, which yielded the HA-Ph microcapsules containing gelation microparticles as template of cavities. The diameter of HA-Ph microcapsules was relatively uniform because the coefficient of variance for the size distribution was 10%. These results demonstrate that HA-Ph microparticles and microcapsules can be obtained using the microfluidic system conventionally used for the preparation of spherical vehicles through HRP-catalyzed reaction[7,10,15] without special concern for the use of HA-Ph.

Effect of Encapsulation on Cells

It was expected that encapsulation process, existence of shear stresses induced by precursor polymers, and time required for encapsulation would alleviate the viability and growth ability of encapsulated cells. We evaluated the effects of encapsulation in

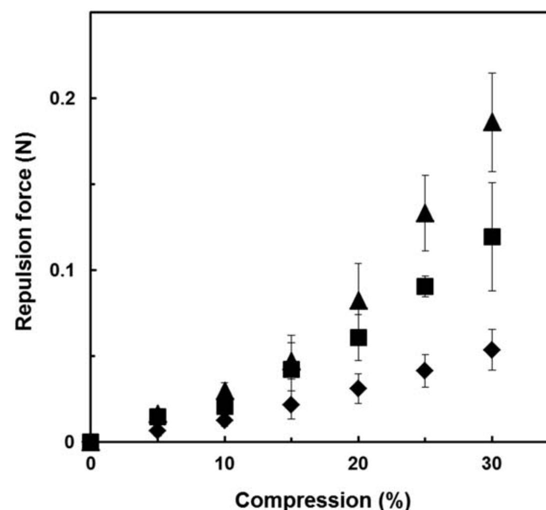

Figure 5. Load-compression profiles of HA-Ph hydrogels prepared at (◆) 30, (■) 60, and (▲) 100 units/mL HRP. Vertical bars: SD (n = 4 or 5).

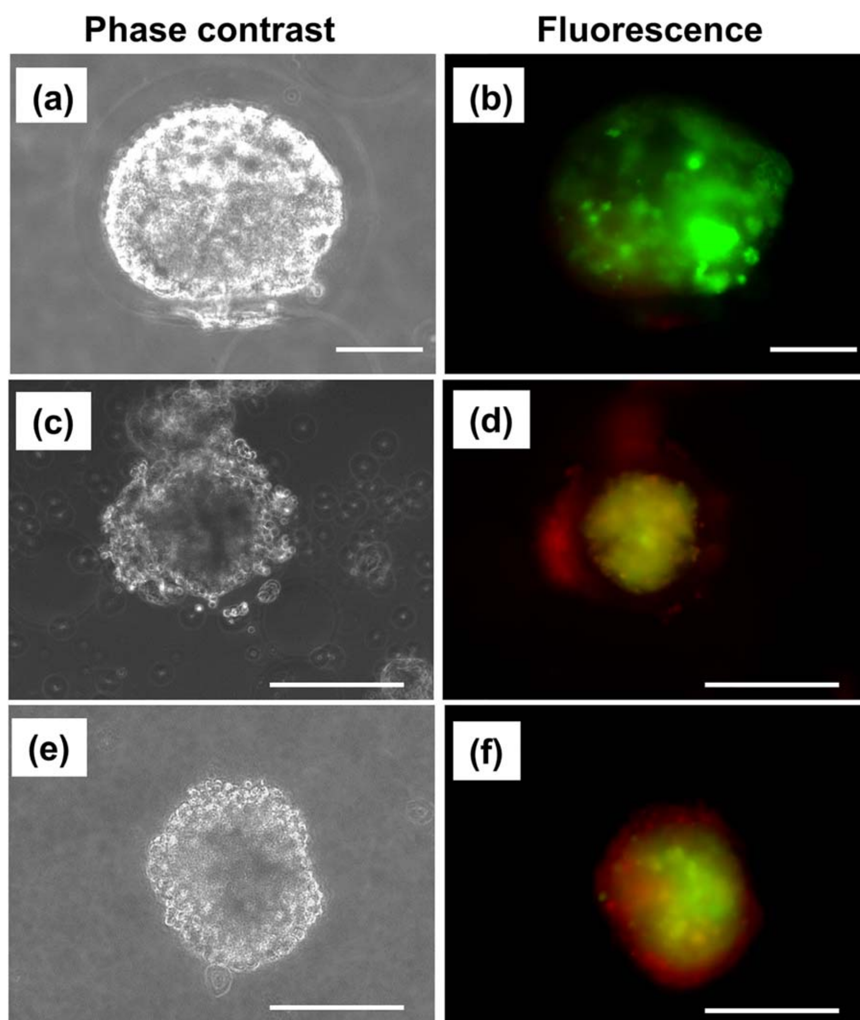

Figure 6. Phase contrast and fluorescence images of HepG2 cells (green) enclosed in HA-Ph microcapsules covered with HeLa cells (red). (a,b) Unmodified surface microcapsules, (c,d) modified surface microcapsules, and (e,f) spherical tissues after degrading HA-Ph membrane of modified surface microcapsules by hyaluronidase. Horizontal bars: (a–b) 100 μm, and (c–f) 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the HA-Ph microparticles and microcapsules, based on the viability of HepG2 cells at 1 h of encapsulation and growth of the cells harvested from these spherical vehicles by degrading the hydrogel using hyaluronidase. Both the spherical vehicles were obtained from the aqueous solution containing 0.75% (w/v) HA-Ph and 100 units/mL HRP. The cell viabilities in the HA-Ph microparticles of 180 ± 20 μm diameter and microcapsules of 200 ± 20 μm diameter with cavities of 150 ± 20 μm diameter were measured by trypan blue dye exclusion and found to be 94.2 ± 2.3 and 97.0 ± 1.1%, respectively. These values were high enough compared with the viability before encapsulation (99.2 ± 0.2%). In addition, morphologies of harvested cells at 1 h were monitored for 108 h and it was found that cells proliferated to a confluent state. The HepG2 cells harvested from HA-Ph microparticles and microcapsules adhered and proliferated on cell culture dishes in the same way as those maintained by conventional subculture and harvested from gelatin microparticles [Figure 3(a–l)]. There was no significant difference in the cell shape between the harvested cells and control cells (free from encapsulation) during the cultures, which indicates the absence of any adverse effect of encapsulation process on the HepG2 cells.

Growth of Encapsulated Cells

The HepG2 cells encapsulated in the HA-Ph microcapsules existed individually in cavities at 4 h of encapsulation [Figure 4(a)]. The cells grew and virtually occupied the cavities by day 8 [Figure 4(b)]. Formation of spherical tissues in individual cavities was confirmed from the remaining of spherical tissues after degradation of the HA-Ph microcapsule membrane using hyaluronidase [Figure 4(c)] and also, the viability of these cells was sufficiently high (93.6 ± 1.5%).

These results indicate that the HA-Ph microcapsules can be used for obtaining spherical tissues in analogy with previously reported Ca-alginate microcapsules having spherical cavities templated by gelatin microparticles.[19] The HA-Ph microcapsules would be useful for transplanting cells and tissues *in vivo*

because the microcapsule membrane will be degraded by hyaluronidase.

The cells in the HA-Ph microparticles obtained from 0.75% (w/v) HA-Ph solution grew slower than those in the microcapsules [Figure 4(d–g)], although the viability of the enclosed cells in the microparticles was relatively high ($85.7 \pm 1.7\%$) at 11 days of encapsulation. This result means that the growth suppression was induced by the HA-Ph hydrogel surrounding HepG2 cells. It is known that the cellular growth is regulated by the microscopic stresses given by the surrounding environment.[7,28] Regarding the effect of HRP concentration, the cells in the HA-Ph microparticles obtained from the solution at a lower content of HRP grew faster [Figure 4(e–g)]. At 11 days of encapsulation, proliferated cells formed clusters in the microparticles obtained at 30 units/mL HRP. In contrast, the cells in the microparticles obtained at 100 units/mL HRP did not form such structure. The slower growth of the cells in the microparticles prepared at a higher content of HRP would be caused by a higher microscopic stress given from surrounding HA-Ph hydrogel.[10] It has been reported that the mechanical properties of the hydrogels obtained through the HRP-catalyzed reaction can be controlled by altering HRP concentration.[14,28,29] In fact, the HA-Ph hydrogel obtained at a higher content of HRP showed a larger repulsion force toward compression (Figure 5). These results demonstrate the feasibility of HA-Ph microparticles for investigating the effect of microscopic stress on cellular behaviors in extracellular matrix. Recently, the stiffness of matrix surrounding cells attracts attention as a regulator of cell behaviors.[21,28] Apart from the cell-enclosing vehicles, the HA-Ph microparticles have a great potential for application in drug delivery because of the biocompatibility and biodegradability of HA-Ph hydrogel.[21] The co-axial microfluidic system is promising for preparing the microparticles containing drug with a narrow distribution in size.[8]

Spherical Tissues with Heterogeneous Cell Layer

The spherical tissues covered with a heterogeneous cell layer would be useful for clinical and pharmaceutical applications.[30–32] By modifying the surface of the microcapsules with gelatin-Ph via HRP-catalyzed reaction, we could develop a cell-adhesive microcapsule surface. As shown in Figure 6(a,b), HeLa cells did not attach on un-modified HA-Ph microcapsules. In contrast, the cells attached and grew on the microcapsule surface soaked in the gelatin-Ph solution containing HRP and H_2O_2 [Figure 6(c,d)]. The difference clearly indicates the possibility of altering the properties of HA-Ph spherical vehicle surfaces by HRP-catalyzed reaction, as reported for Alg-Ph spherical vehicles.[27] After establishing a HeLa cell layer on the modified microcapsules, we treated them with hyaluronidase. The HeLa cell layer began shrinking after the treatment without loss of layer structure, and the spherical tissues of HepG2 cells covered with the HeLa cell layer could be obtained [Figure 6(e,f)]. At 1 h of treatment, dissociation of the outer HeLa cells did not occur when the tissues were manipulated using a pipette.

CONCLUSIONS

In this work, we aimed to examine the possibility of preparation of cell-enclosing HA-based microparticles and microcapsules via the HRP-catalyzed reaction using a microfluidic system. For this purpose, we employed the HepG2 cells as a model cell line. The spherical vehicles were obtained by extruding the HA-Ph aqueous solution containing HRP and cells or cell-enclosing gelatin microparticles into the flow of liquid paraffin containing H_2O_2. Diameter of the vehicles was controllable between 100 and 300 μm by changing the flow rate of liquid paraffin. The HepG2 cells enclosed in the vehicles kept more than 90% viability and grew on a cell culture dish after being harvested from the vehicles with hyaluronidase. The cells in the microcapsules grew faster than those in the microparticles and formed spherical tissues in the cavities. Using the microcapsules, spherical tissues of HepG2 cells covered with a heterogeneous cell layer could be prepared by degrading the microcapsule membrane with hyaluronidase after the establishment of cell-adhesive surface and HeLa cell layer. The cells enclosed in the microparticles obtained from the solution with a higher content of HRP grew slower. These results demonstrate the possibility of preparation of cell-enclosing HA-based microparticles and microcapsules using our microfluidic system. Considering the superior biocompatibility and biodegradability of HA-Ph, we can conclude that these vehicles have a huge potential of applications in a biomedical field.

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