Peroxidase-Catalyzed Cell Encapsulation in Subsieve-Size Capsules of Alginate with Phenol Moieties in Water-Immiscible Fluid Dissolving H_2O_2

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Introduction

For decades, enzymatic polymerization in vitro has been extensively studied because specific enzyme catalysis provides a novel synthetic route for functional and useful polymeric materials.[1,2] In particular, peroxidase has been frequently used for the oxidative polymerization of phenol derivatives under mild reaction conditions.[3,4] In the reaction, peroxidases function as oxidoreductases that catalyze the oxidation of donors using H_2O_2 resulting in polyphenols linked at the aromatic ring by C−C and C−O coupling of phenols. Recently, we developed alginate with phenol moieties (Alg−Ph) and showed that a 1.0% (wt/vol) solution of Alg−Ph, containing 2.8 phenols per 100 repeat units of alginate gelated via either formation of cross-links by calcium ions or horseradish peroxidase (HRP)-catalyzed oxidative coupling of phenols using H_2O_2 dissolved in the aqueous solution or both.[5] In addition, we revealed that the oxidative coupling of the phenols in the alginate gel cross-linked by Ca^{2+} was effective for suppressing the destabilization of the gel resulting from a loss of bonding between the Ca^{2+} and alginate. The destabilization has been a problem in the field of cell encapsulation in which the spherical alginate gels cross-linked by Ca^{2+} have been the most frequently investigated as cell-enclosing vehicles.[6]

Encapsulation of mammalian cells in physical membranes has been investigated since 1964[7] as a basic research tool, for production of various useful biomolecules such as recombinant proteins and enzymes by in vitro cultivation, and as therapeutic devices for tissue and cell transplantation toward a variety of diseases such as diabetes and cancer.[8,9] Recently, we reported a technique that enables preparation of mammalian cell-enclosing capsules of less than 100 μm in diameter, termed "subsieve-size capsules".[2] The size is about one-tenth that of conventional cell-enclosing capsules (500−1000 μm). Mammalian cell-enclosing capsules of less than 100 μm in diameter and with narrow size distribution have not been reported except for our reports.[10−13] Such a reduced size resulted in enhancements of mechanical stability and molecular exchangeability between the enclosed cells and the ambient environment.[11] In addition, fibrous cellular formation around capsules after implantation in vivo is suppressed by the reduction in size.[12] The subsieve-size capsule preparation technique involves a process of droplet breakup in a coflowing water-immiscible fluid via formation of a thin jet of an aqueous polymer solution (coflowing method).[10,13] Compared with the well-known emulsification techniques that result in subsieve-size droplets using a magnetic stirrer and a homogenizer,[14,15] the droplets produced by the coflowing method show a narrow distribution in size.[10] In addition, the emulsification process of this method scarcely hinders the viability of mammalian cells.[10]

To date, we have successfully prepared subsieve-size capsules from agarose via a thermal gelation process using the coflowing method.[11,16,17] Thermal gelation is simple and suitable for gelation of an aqueous polymeric solution in a water-immiscible fluid. In this study we aimed to develop mammalian cell-enclosing subsieve-size capsules from the Alg−Ph via the coflowing method. Compared with agarose, attractive features of alginate as a material for cell encapsulation is that they have carboxyl groups enabling modification using cationic polymers via formation of electrostatic bonds for further enhancement of biocompatibility, immunoisolatability, and mechanical stability.[6,18−20] The ionically and enzymatically cross-linkable Alg−Ph retains more than 90% of original carboxyl groups.[5] One well-known method for obtaining alginate capsules in a water-immiscible fluid is on the basis of coalescence between droplet(s) of aqueous alginate solution and droplet(s) of an aqueous divalent cation solution such as calcium ions. Sugiura et al. prepared cell-enclosing alginate capsules of ca.150 μm in diameter on the basis of the coalescence of droplets prepared using an array of micronozzles of 30 μm × 30 μm.[21] In their report the droplets had high sphericity before gelation. However, the resultant gelated capsules were far from a spherical shape. Such an awkward shape results in problems in mechanical stability. The difference in sphericity before and after the coalescence of droplets for gelation shows a drawback of the method. In this study we designed a process in which H_2O_2 is supplied via molecular diffusion from the water-immiscible fluid surrounding the droplets of aqueous Alg−Ph solution, which contain mammalian cells and HRP. The cell-suspending solution gelates only after contacting the ambient fluid. To provide evidence of the feasibility of this process and the subsieve-size Alg−Ph capsules we determined the applicability of the coflowing method, the swelling behavior of the resultant capsules, and the influence of the capsule preparation process on mammalian cells.

Materials and Methods

Materials. Sodium alginate with a high content of guluronic acid (molar ratio of mannuronic acid to guluronic acid of 0.65) and a molecular weight of 70 000 was kindly donated by Kimica (Tokyo, Japan). Tyramine hydrochloride was purchased from Sigma (MO). Aqueous H_2O_2 solution (31%) and liquid paraffin were obtained from Kanto Chem. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Peptide Inst. (Osaka, Japan). Horseradish peroxide (HRP, 170 units/mg), hydroxysulfosuccinimide (NHS), and lecithin from soybean were obtained from Wako Chem. (Osaka, Japan). Crandall-Reese feline kidney cells (CRFK cell line) were grown in Dulbecco's modified Eagle's Medium (DMEM, Sigma, MO) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 400 mg/dL glucose, 75 mg/L penicillin, and 50 mg/L streptomycin and cultured in a humidified atmosphere at 37 °C with 5% CO_2.

Synthesis of Alg−Ph. Both ionically and enzymatically cross-linkable alginate with phenol moieties was synthesized by the method reported previously.[5] Briefly, sodium alginate was dissolved in deionized water at 1.0% (wt/vol). The solution was mixed with the same

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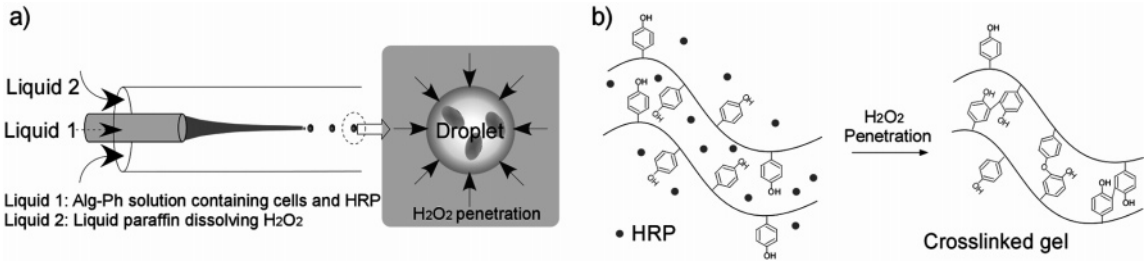
Scheme 1. Schematic Illustrations of (a) the Breakup of Cell-Enclosing Droplets in a Coflowing Stream of Liquid Paraffin Containing H_2O_2 and HRP-catalyzed Gelation and (b) HRP-Catalyzed Cross-Link Formation Using H_2O_2 Penetrating from the Ambient Liquid Paraffin



volume of deionized water containing 50.4 mM EDC, 25.2 mM NHS, and 9.0% (wt/vol) tyramine hydrochloride. The mixture was gently stirred for 20 h at 25 °C, and the resulting polymer was dialyzed (molecular weight cutoff = 10 000−20 000 Da) against deionized water for 2 days with six changes of the dialysis solution. The sample was then lyophilized. The resultant polymer contained 2.8 phenols per 100 repeat units of alginate.

Preparation of Phenol−Alginate Capsules. To dissolve H_2O_2 in liquid paraffin, 5 mL of aqueous 31% (wt/wt) H_2O_2 solution was mixed with 1000 mL of liquid paraffin and vigorously stirred using a magnetic stirrer for 12 h at room temperature. Then, the emulsion was centrifuged at 2000 rpm for 10 min to separate liquid paraffin and aqueous H_2O_2 solution. The concentration of H_2O_2 in the resultant liquid paraffin calculated from the concentrations in aqueous solution before and after mixing with liquid paraffin was 0.82 mmol/L. Lecithin was dissolved at 3.0% (wt/wt) in the collected liquid paraffin containing H_2O_2. The solution was used as the ambient fluid in the enzymatic capsule production process (Scheme 1). Alg−Ph was dissolved at 1.5% (wt/vol) in calcium-free Krebs Ringer Hepes buffered solution (CF-KRH, pH7.4) containing 1.6 units/mL of HRP. This Alg-Ph solution was extruded from a 26-gauge needle into a coflowing immiscible stream of the liquid paraffin containing H_2O_2 at 0.1 mL/min. A droplet generator designed in our laboratory was used for this process.[16] The liquid paraffin suspending partially gelated capsules was collected in a 50 mL plastic tube. After 10 min of standing, KRH was added to the tube. The tube was then centrifuged at 2500 rpm for 2 min to allow collection of the capsules.

Diameter Change in Solvents. Immediately after collection of capsules via centrifugation, 0.15 mL of enzymatically gelated capsules was soaked in 15 mL of 0.9% (wt/vol) NaCl, simulated body fluid (SBF), DMEM containing 10% (vol/vol) FBS, or 55 mM sodium citrate buffered with 10 mM Hepes (pH 7.4) to determine the swelling behavior of the capsules under the conditions of practical use. These solutions were renewed twice every 4 h and subsequently renewed every 24 h for 7 days. The SBF originally developed by Oyane et al. contained 142.0 mM Na^+, 103.0 mM Cl^-, 10.0 mM HCO_3^-, 5.0 mM K^+, 1.5 mM Mg^{2+}, 2.5 mM Ca^{2+}, 1.0 mM PO_4^{2-}, and 0.5 mM SO_4^{2-}. Except for HCO_3^-, these ion concentrations are the same as those in human plasma.[22] Alg−Ph capsules of 233 ± 23 μm in diameter were used for this study. The capsule diameter change ratio at each incubation period was the value normalized to the capsule diameter just after collection from liquid paraffin. The diameter of capsules represents a mean value from more than 50 capsules.

Cell Studies. Cell-enclosing subsieve-size capsules were prepared from the 1.5% (wt/vol) Alg−Ph solution containing CRFK cells at 1.5 × 10^7 cells/mL under the condition determined on the basis of the result obtained in the study described above. To evaluate the cytotoxicity of the encapsulation process, the cell-enclosing capsules were soaked in culture medium containing alginate lyase (0.05 mg/mL, Alginate lyase S, Nagse Chemtex, Osaka, Japan) at room temperature after 30 min of encapsulation. The capsules were completely degraded after 10 min of incubation, and viability of the cells was measured by trypan blue exclusion using a hematocytometer. To determine the possible occurrence of harmful effects that require time to induce cell death and growth, the cells recovered from subsieve-size capsules were seeded at 3.0 × 10^5 (viable cells)/well in 60-mm tissue culture dishes. Their proliferation profiles were measured using a hemocytometer. Cells that had not been subjected to the capsule preparation process (i.e., cells prepared on the basis of a normal subculture protocol using trypsin-EDTA solution) were used as controls. The growth profile of enclosed cells was estimated based on the increase in the amount of water-soluble formazan dye, derived from tetrazolium salt dissolved in the medium suspending cell-enclosing capsules, using a colorimetric assay kit (Cell counting kit-8, Dojindo, Kumamoto, Japan), indicating the presence of dehydrogenase within intact mitochondria in living cells and therefore the physiological state of the enclosed cells. In short, 400 μL of the medium suspending cell-enclosing capsules was poured into 24-well cell culture dish, and then 40 μL of the reagent in the assay kit was added into the well. After 4 h of incubation at 37 °C, the absorbance at 450 nm was measured using a spectrophotometer.

Statistical Analysis. Comparisons between the two groups were made using a two-tailed Student's unpaired *t* test. A value of $p < 0.05$ was considered to be significant.

Results and Discussion

Capsule Preparation. Features of the process that we attempted to develop in this study are (1) using alginate with phenol moieties, (2) preparing subsieve-size droplets from an aqueous solution using a coflowing method, and (3) gelation of subsieve-size droplets via an HRP-catalyzed oxidative reaction using H_2O_2 supplied from the ambient water-immiscible fluid of liquid paraffin. At first we studied whether subsieve-size capsules can be prepared from aqueous Alg−Ph solution via this process. The diameters of the resultant enzymatically gelated capsules decreased with increasing flow rate of the ambient liquid paraffin as shown in Figure 1. At 2.5 mL/min of liquid paraffin flow the capsule diameter was 283 ± 36 μm. This value decreased with increasing a flow rate of liquid paraffin, and subsieve-sized capsules of 80.8 ± 12.0 μm were obtained at a flow rate of 20.0 mL/min of liquid paraffin. The tendency of capsule diameter to be reduced by an increase in

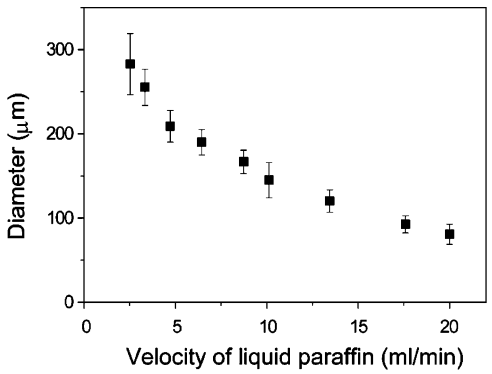


Figure 1. Alg−Ph capsule diameter as a function of the velocity of liquid paraffin. 1.5% (wt/vol) aqueous Alg−Ph solution containing 1.6 units/mL HRP was extruded from a 26-gauge needle at 0.1 mL/min. Error bars represent standard deviation of more than 50 capsules.

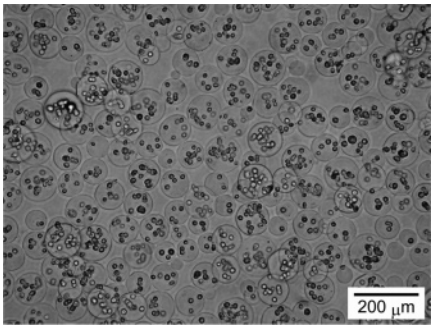


Figure 2. Micrograph of CRFK cell-enclosing Alg−Ph capsules of 84.8 ± 13 μm in diameter. The dark particles of ca. 10 μm in diameter in transparent particles represent individual enclosed cells.

the ambient fluid flow is in agreement with studies of aqueous solutions of unmodified alginate[10] and agarose.[11] No particles were obtained following extrusion of a 1.5% (wt/vol) unmodified alginate solution containing HRP at 1.6 units/mL into liquid paraffin containing H_2O_2; similarly, no particles were obtained following extrusion of 1.5% (wt/vol) Alg−Ph solution containing no HRP into the same solution. These results clearly show the requirement for formation of HRP-catalyzed cross-linking of phenols in Alg−Ph using the H_2O_2 supplied from ambient liquid paraffin as well as the applicability of the coflowing method for obtaining subsieve-size droplets from Alg−Ph solution. In addition, Figure 2 shows that the resultant capsules had high sphericity. This high sphericity shows that the sufficient degree of enzymatic gelation necessary for fixing the final shape of gel does not occur instantaneously. In the case of using aqueous calcium chloride solution as an ambient fluid, the gel we obtained was not a spherical particle but a continuous fiber due to the instantaneous gelation of alginate solution after contacting the calcium ions in the ambient solution.

Swelling Property. As a next step we investigated the swelling properties of the enzymatically gelated capsules. Swelling of capsules results in mechanical instability. For cell entrapment applications aimed at implantation in vivo the mechanical properties of cell-enclosing vehicles are important because they must possess sufficient mechanical strength to withstand compressional forces from the surrounding tissues in vivo without fear of deformation or collapse. For production of useful proteins using a bioreactor suspending cell-enclosing capsules, breakage of cell-enclosing capsules exposes the cells to shear stress resulting from the flow of medium. Mammalian cells are easily damaged by forces exerted from the external environment, such as shear stress. It is known that the most frequently investigated alginate gels gelated by calcium ions swell in body fluid and cell cultivation medium until the achievement of an equilibrium state.[23,24] This swelling results from the exchange of cross-linking calcium ions with non-gel-inducing sodium ions in the ambient fluid. The enzymatically gelated capsules showed no change in diameter after being soaked in 0.9% (wt/vol) NaCl solution or the calcium chelating agent solution 55 mM sodium citrate for 7 days of study ($p > 0.51$ at day 7 vs immediately after capsules preparation, Figure 3). These results are reasonable because multivalent cations were not responsible for gelation of the Alg−Ph solution in the capsule generation process of this study.

Interestingly, a reduction in capsule diameter was detected in capsules soaked in SBF: after 1 day of soaking, the diameter decreased to 90% of that before soaking ($p < 0.001$). An additional 1 day of soaking resulted in a further reduction in diameter to ~87% of the original diameter ($p = 0.03$ vs day 1). The value remained unchanged for the following period to

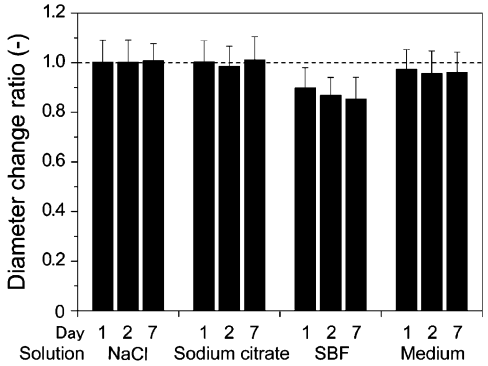


Figure 3. Diameter change ratios of enzymatically gelated Alg−Ph capsules nonenclosing cells after soaking in 0.9% (wt/vol) NaCl, 55 mM sodium citrate, SBF, or DMEM medium containing 10% (vol/vol) FBS. The diameter at each incubation period was normalized to the diameter immediately after preparation of capsules. Error bars represent standard deviation of six separate sets of experiments.

day 7 ($p = 0.32$ vs day 2). The diameter reduction during the first 2 days of soaking can be explained by formation of cross-links between the carboxyl groups of Alg−Ph and calcium ions in SBF. The Alg−Ph used in this study contained only 2.8 phenols per 100 repeat units of alginate; that is, the majority of the carboxyl groups in native alginate remained in Alg−Ph. This means that the capsules can be modified by cationic polymers such as poly-L-lysine and chitosan with subsequent modification with anionic polymers via electrostatic interaction for enhancements of immunoisolatability, mechanical stability, and biocompatibility.[18−20] The steady value of diameter after 2 days of soaking, despite renewing the soaking solution every 24 h, was interpreted as a consequence of having achieved an equilibrium state of ion exchange between the alginate and the ambient fluid. Our previous finding[24] regarding the swelling behavior of alginate gel cross-linked by calcium ions in SBF supports this interpretation: the degree of swelling showed a constant value after several days of soaking. The diameters of capsules soaked in medium also decreased. By day 7 the diameter was reduced to 96.1% of that before soaking ($p = 0.01$). The degree of diameter reduction in medium was smaller than that in SBF ($p < 0.001$ at day 7). This difference is due to the difference in the concentrations of multivalent cations, e.g., Ca^{2+}, non-gel-inducing cations, e.g., Na^+, and multivalent cations chelating ions, e.g., PO_4^{2-} in each solution.

Cell Encapsulation. The ultimate purpose of this study is development of cell-enclosing capsules for bioproduction and biomedical applications. Viability of the enclosed cells is extremely important for these applications. Thus, investigations into the influences of the gel surrounding the cells and the method of capsule preparation process on cells are essential for revealing their feasibility. In a previous study we showed that the coflowing method scarcely hinders the viability of the enclosed cells. The novel process of this study involves enzymatic gelation using H_2O_2 supplied from the ambient water-immiscible fluid. The H_2O_2 was a potential candidate for hindering the viability of cells. In order to study its effect on cell viability, we first investigated cells recovered from subsieve-size capsules by enzymatically degrading the cell-enclosing Alg−Ph capsules using alginate lyase. The viability of the cells after 30 min of encapsulation was 91.8 ± 3.2% ($n = 7$). This value was almost the same as that measured for the cells enclosed in nongelated subsieve-size droplets of sodium alginate solution.[10] In addition, we could not detect any harmful effects by observation of the morphology of the recovered cells on a tissue culture dish. The recovered cells adhered and spread on

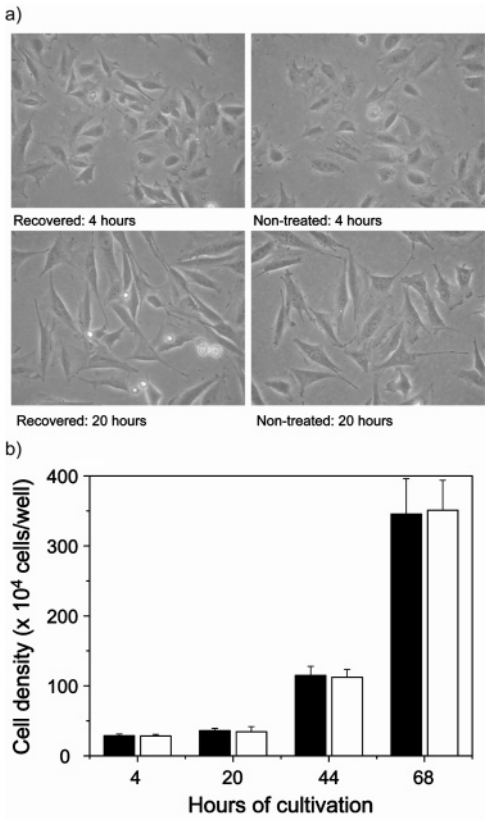


Figure 4. (a) Micrographs of CRFK cells cultured on tissue culture dishes of 60 mm in diameter at 4 and 20 h after seeding. Recovered: cells seeded after being recovered from Alg−Ph capsules by degradation using alginate lyase. Nontreated: cells seeded on the basis of a general subculture protocol. Cells were seeded at 3.0×10^5 viable cells/well. (b) Proliferation profiles of CRFK cells on tissue culture dishes. Columns indicate mean densities of (black) recovered cells and (white) nontreated cells. Error bars represent standard deviation of four separate sets of experiments.

the tissue culture dish in the same way as cells seeded on the basis of general subculture protocol, i.e., cells not subjected to the capsule preparation process (Figure 4a). No significant difference was detected in the growth profiles of these two groups of cells (Figure 4b, $p > 0.63$), and the cells in both groups achieved confluence after 68 h of observation. From these studies we could not find any harmful effect of the novel enzymatic encapsulation process on the surface membranes of cells or their viability despite the existence of H_2O_2 in the reaction system. Because the toxic effect of H_2O_2 on cells is well known, this promising result regarding cytotoxicity was unexpected at the start of the study. Indeed, cells exposed to CF-KRH containing 2.7 mM H_2O_2 for 3 h showed no mitochondrial activity in our preliminary study. A gradual and slight quantity of penetration of H_2O_2 from ambient liquid paraffin into the droplets dissolving HRP and the immediate consumption of the H_2O_2 by the HRP-catalyzed oxidative reaction is a possible explanation of these results. Figure 5 shows that the mitochondrial activity of cells per capsule gradually increased with increasing cultivation period and achieved 9.3-fold after 63 days of cultivation, compared to day 1. As well as this transition of mitochondrial activity, Figure 6 clearly shows growth of cells in the Alg−Ph capsules. The majority of capsules contained cellular clumps of 20−70 μm in diameter at the end of the cultivation period. These results show the Alg−Ph gel gelated via an HRP-catalyzed reaction allows growth of enclosed cells as the same with unmodified alginate gel cross-linked by multivalent cations.[25]

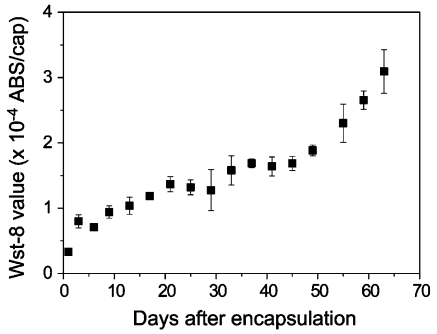


Figure 5. Mitochondrial activity of enclosed cells per enzymatically gelated conjugate capsules. Error bars represent standard deviation of three separate sets of experiments.

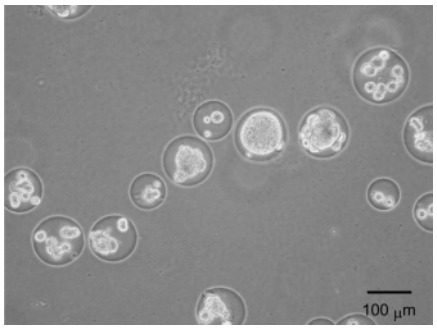


Figure 6. Micrograph of CRFK cell-enclosing conjugate capsules after 65 days of cultivation.

We also attempted to prepare cell-enclosing capsules of more than 400 μm in diameter using the same process. However, more than 30 min of soaking in liquid paraffin with H_2O_2 for enzymatic reaction was necessary for this. The time necessary for obtaining gelated capsules increased with increasing diameter of droplet. This finding shows that diffusion of H_2O_2 from liquid paraffin is slow and small. Sufficient oxygenation is needed for survival of cells; thus, a shorter time of suspending in liquid paraffin is preferable. These findings indicate that the novel process developed in this study is more suitable for subsieve-size capsule production.

Conclusions

Peroxidase-catalyzed oxidative coupling of phenols proceeds under very mild conditions suitable for mammalian cells except for the necessity of H_2O_2 for the reaction. Uyama and coworkers expanded the potential utility of this reaction to enable polymerization of macromolecules and gelation of polymers.[3,4] In this study we applied this methodology to prepare subsieve-size cell-enclosing alginate capsules in a coflowing water-immiscible fluid. We successfully suppressed the potentially harmful effects of H_2O_2 by a novel process that uses liquid paraffin containing H_2O_2 with a sufficient quantity for the HRP-catalyzed gelation of subsieve-size droplets but an insufficient quantity to hinder the viability of cells. The resultant enzymatically gelated gel did not possess the drawback of conventional alginate gels cross-linked by calcium ions: conventional alginate gels swell in body fluid due to the exchange of cross-linking calcium ions with non-gel-inducing sodium ions. More than 90% of the cells enclosed in the enzymatically gelated capsules were kept alive after the encapsulation process and proliferated in the capsule for 63 days of study. These results demonstrate the feasibility of the process of enzymatic subsieve-size capsule generation in a water-immiscible coflowing stream and of enzymatically gelated Alg−Ph capsules as cell-enclosing vehicles toward bioproduction and biomedical applications.

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