Bromo-oxidation reaction in enzyme-entrapped alginate hollow microfibers

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In this article, the authors present the fabrication of an enzyme-entrapped alginate hollow fiber using a microfluidic device. Further use of enzyme-entrapped alginate hollow fibers as a biocatalytic microchemical reactor for chemical synthesis is also deliberated in this article. To ensure that there is no enzyme leaching from the fiber, fiber surfaces were coated with chitosan. To confine the mobility of reactants and products within the porous hollow fibers the entire fibers were embedded into a transparent polydimethylsiloxane (PDMS) matrix which also works as a support matrix. A vanadium-containing bromoperoxidase enzyme isolated from *Corallina confusa* was used as a model enzyme to demonstrate the use of these alginate hollow-fiber reactors in bromo-oxidation of phenol red to bromophenol blue at different dye flow rates. Stability of the entrapped enzyme at different temperatures and the effect of the chitosan coating on the reaction conversion were also studied. It was observed that molecules as big as 27 kDa can be retained in the matrix after coating with chitosan while molecules with molecular-weight of around 378 Da can still diffuse in and out of the matrix. The kinetic conversion rate in this microfluidic bioreactor was more than 41-fold faster when compared with the standard test-tube procedure. © 2011 American Institute of Physics. [doi:10.1063/1.3605512]

I. INTRODUCTION

The use of enzyme-catalyzed reactions in complex chemical reactions is one of the future solutions that embrace greener and sustainable technologies. This has led to a worldwide interest and evolution of biocatalysts.¹ However, for nonbiological applications such as organic syntheses, analytical chemistries and therapeutics, enzymes and cells may be unstable, difficult to handle, and not reusable. This limits the use of such biocatalysts on an industrial scale. To overcome this problem, it is important that the cell or enzyme is immobilized; the processes used for such immobilization should be straightforward, cost-effective, and the immobilized product should be suitable for long-term use. Several immobilization methods have been described in the literature, including covalent attachment, cross-linking, microencapsulation, and entrapment in beads and fibers. Several types of support or matrix materials for enzyme immobilization have been de-

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scribed, which include organic natural polymers such as cellulose, dextrans, agar, agarose, chitin, alginate, collagen and albumin.² Among the various immobilization methods, entrapment of enzymes and cells in alginate beads or fibers has attracted much attention due to its potential and excellent capabilities for enzyme or cell encapsulation, in states reminiscent of natural matrices. Even though there are several other ways to improve enzyme stability such as multipoint and multisubunit immobilization, however, both the method requires highly active support for covalent bonding, hence limiting the possibility of reuse of enzyme. In addition, multisubunit immobilization is more suitable for multimeric enzymes.^{3–7} On the other hand, encapsulation of enzyme in a semipermeable or porous matrix not only ensures stability of enzyme in its free state but also allows possibility of reuse by extracting the encapsulation matrix. Microfluidic systems have been the preferred way to prepare alginate fibers because of the inherent simplicity in the fabrication process.^{8–10} The use of hollow fibers for encapsulation of biological materials holds tremendous untapped potential. Selectively permeable hollow fibers have already been employed in various industrial applications such as industrial waste water treatment¹¹ using a so-called extractive membrane bioreactor (EMBR)^{12,13} and in ultrafiltration. Additionally, hollow fibers have diverse potential applications in hemodialysis,¹⁴ neuronal cell culture,¹⁵ and tissue engineering.^{16–18} The use of cell culture hollow-fiber bioreactors is well established in the production of monoclonal antibodies, recombinant proteins, viruses and viral antigens, and viable cells.^{19–21} Despite the tremendous scope of hollow microfiber applications in biochemical and chemical syntheses, the use of such fibers in biocatalytic syntheses of organic chemicals has not been utilized because fabrication of such fibers remains a challenge. Recently, Sugiura *et al.* synthesized alginate hollow fibers in a three-step method consisting of solid fiber fabrication using a microfabricated nozzle array; the fiber was coated with poly-L-lysine and the core alginate removed with sodium citrate.¹⁰ We have previously proposed a simple one-step method for “on-the-fly” fabrication of hollow microfibers using a microfluidic chip²⁰ and demonstrated the use of such fibers in cell coculture. Even though this method was simple, cost effective and permitted easy microfiber size adjustment, protein or enzyme loading/entrapment onto microfibers walls remains a challenge.²⁰ Here, for the first time, we report a simple fabrication method for enzyme-entrapped in the walls of alginate hollow microfibers using a microfluidic device, and hence the capability of using these fibers as a microbioreactor. These enzyme-entrapped hollow microfibers were embedded in a PDMS mold after coating of chitosan to produce a microfluidic based biocatalytic reactor. We can control the size of fiber (120–250 μm) by adjusting the flow rates of the core and sheath fluids, as described briefly in the experimental section.

There are several research articles on the application of microfluidics in the field of microreaction technology. However, the applications of biocatalytic or enzymatic microreactors are limited to chemical analysis, such as work of Nien *et al.* where they have used an enzyme-trapped polymeric electrode as a sensor for glucose in a microfluidic chip.²² The microfluidic based enzyme reactors are still in its initial stage of development and are not being adequately explored due to instability, difficulty in handling and poor reusability of cells or enzymes. The works done by Srinivasan *et al.* and Lukarift *et al.* are the only published cases of the use of enzyme-catalyzed synthetic chemistry. Srinivasan *et al.* used the microfluidic devices to carry out the enzyme-catalyzed oxidative polymerization of phenols,²³ while Lukarift *et al.* used a microfluidic device to carry out a two stage synthesis where one of the stages was an enzyme-catalyzed polymerization of 2-aminophenoxazin-3-one (APO).²⁴ In this work, we have employed a unique marine metalloenzyme vanadium-containing bromoperoxidase (VBPO) enzyme, isolated from *Corallina confuse*, trapped in an alginate hollow microfiber as microfluidic bioreactor as a model to demonstrate the bromo-oxidation of Phenol Red to Bromophenol Blue. The vanadium bromoperoxidase (VBPO) is known to catalyze the oxidation of chloride, bromide, and iodide in presence of hydrogen peroxide and biosynthesis of halogenated organic compounds.^{25,26} Vanadium (V) is an essential cofactor for the catalytic activity of VBrPO; however, the vanadium site in VBrPO is largely inaccessible by conventional spectroscopic techniques.²⁷

II. MATERIALS AND METHODS

A. Materials

High gel-strength, medium-viscosity sodium alginate (Manugel GMB) was obtained as a kind gift from ISP Alginates (Surrey, UK) and was used to fabricate the enzyme-entrapped hollow microfibers. Chitosan was purchased from Wako (Chitosan 1000 (10 kDa), Osaka, Japan), sodium triphosphate pentabasic, calcium chloride, phenol red, bromophenol blue, potassium bromide, acetic acid, sodium acetate, ammonium sulfate and ammonium metavanadate were from Sigma-Aldrich (St. Louis, MO). Sodium citrate was from Fluka (St. Louis, MO) and 4-morpholine ethane-sulfonic acid (MES) was from Merck (Whitehouse Station, NJ) and hydrogen peroxide (Samchun Chemicals, Seoul, Korea) was used as received.

B. Enzyme extraction

Corallina confusa was collected in spring 2008 in Sodol, Kangleung, South Korea. The alga was transported directly from the beach to Chungnam National University where it was transferred into aerated sea water tanks and maintained at 4 °C. The vanadium bromoperoxidase (VBPO) enzyme was isolated using a method described elsewhere²⁸ for enzyme isolation from *Corallina officinalis*. Briefly, *Corallina confusa* cells were first ground in acetate buffer (1M, pH 5.5; 2 ml/g of alga). Solid debris was removed by centrifugation and the supernatant was subjected to ammonium sulfate fractionation. The 30%–60% ammonium sulfate precipitate, containing most of the VBPO, was dissolved in MES buffer (50 mM, pH 6.5) with 10 μ M ammonium meta-vanadate, which saturated the enzyme active site during storage. This solution was then dialyzed against MES buffer (50 mM, pH 6.5) using a pleated dialysis tube (“Snake Skin,” molecular cutoff 10 000; Pierce, Rockford, IL) and the insoluble protein was removed by centrifugation. The supernatant was stored at 4 °C until use.

C. VBPO

The VBPO assay was based on the method of de Boer *et al.*,²⁹ and involves bromination of Phenol Red to Bromophenol Blue by monitoring the conversion at 580 nm. In brief, each assay contained 2 ml of 100 μ M phenol red, 0.2 ml of 1.0 M potassium bromide, 0.2 ml of 10 mM hydrogen peroxide and enzyme (0.1 ml). At intervals of 1 min, absorbance was measured using an AvaSpec-3648 Fiber Optic Spectrometer (Eerbeek, The Netherlands), scanning the absorbance between 350 and 650 nm. The enzyme concentration was 4.5 units/ml.

D. Fabrication of alginate hollow fibers

Figure 1(a) is a schematic of hollow microfiber generation and also shows the microfluidic device used. Fabrication of the microfluidic device was carried out as described in our previous reports.^{30,31} In brief, a PDMS platform with a preformed principal hole (1 mm inner diameter) was employed and pulled borosilicate glass micropipettes were used. A photograph of the working microfluidic device containing hollow microfibers is shown in Fig. 1(b). For the fabrication of alginate hollow microfibers, two fluids (2% [w/v] sodium alginate for sheath flow and 100 mM CaCl_2 for core flow) were introduced into the sheath and core inlet ports, respectively, using two syringe pumps. A three-dimensional (3D) coaxial sample flow stream of alginate, around the core flow of CaCl_2 solution, was formed at the point where the two flows merged. At the fluid interface, the sodium alginate solution met the polycationic (Ca^{2+}) material and gelation of cylindrical hollow fibers was achieved by diffusion-controlled ionic cross-linking. Finally, fibers were collected in a beaker containing 100 mM CaCl_2 . To generate the fibers of different internal diameters, only the core flow rate (0.42, 0.64, and 0.88 ml/min) were changed while the sheath flow rate (0.05 ml/min) was kept fixed. To create enzyme-entrapped fibers, an equal volume of enzyme extract was mixed with 4% (w/v) alginate solution to achieve a 2% (w/v) alginate polymer with 4.5 units/ml of enzyme. The prepared hollow fibers were coated with 4% (w/v) chitosan solution,

FIG. 1. (a) Schematic of hollow microfiber generation using a two-channel microfluidic device. (b) Photograph of the microfluidic device showing generation of alginate microfibers composed of 1% (w/v) Na-alginate mixed with blue dye. (c) Enzyme-entrapped alginate hollow fibers.

by dip coating, to prevent seepage of immobilized enzyme alginate hollow fiber from the alginate matrix. Finally, the fiber was embedded into half-cured PDMS solution and subsequent curing was carried out on a hot plate for 1 h at 70 °C.

E. Bulk/batch reaction conditions

The reaction mixture was composed of 0.025 mM Phenol Red, 0.5 mM potassium bromide, and 0.25 mM hydrogen peroxide in 50 mM MES buffer, pH 6.5. Reactants were thoroughly mixed and 0.1 ml of enzyme solution was added to start the reaction. Reactions were monitored at 1 min intervals, with subsequent scanning of the absorbance at 350–650 nm. With continued Bromophenol Blue production, the peak at 590 nm increased continuously until the reaction was complete at 170 min (Fig. 2).

III. RESULTS AND DISCUSSION

A. Generation of alginate hollow fiber

As shown in Fig. 1(b), the successful generation of hollow alginate microfibers is performed by the coaxial microfluidic device [Fig. 1(a)]. The alginate hollow fibers were highlighted using a blue dye (Papicel Red IJ-F3B; Eastwell Co. Ltd., Seoul, Korea) that stains the alginate. In initial experiments, hollow alginate microfibers were formed without enzyme. At a fixed sheath flow condition, based on the increase of the core flow rates, the diameters of the hollow fiber were increased by approximately 45%. Also, to demonstrate a symmetric hollow-fiber shape, fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) was loaded and immobilized into the outer-wall of the alginate hollow fibers and imaged using confocal laser scanning microscopy (LSM-510 META instrument; Carl Zeiss, Darmstadt, Germany), scanning electron microscopy

FIG. 2. Absorbance spectra of conversion of phenol red to bromophenol blue in the bulk reaction.

(SEM) (S-4700 instrument; Hitachi Co., Japan), and optical microscopy (Axiovert 200M instrument; Carl Zeiss). The fluorescence, SEM, and optical images in Fig. 3 clearly show the generation of hollow fibers.

FIG. 3. (a) Fluorescence micrograph of BSA-FITC-immobilized alginate hollow fiber by using Z-stack scanning. (b) Fluorescence image of alginate hollow fiber showing bore of the fiber. (c) SEM image of a dried alginate hollow fiber. (d) Alginate fiber with brominated product.

FIG. 4. Enzyme-entrapped alginate hollow microfibers embedded in a PDMS matrix. (a) Fabrication schematic. (b) Photograph of working device.

B. Embedding fiber for reactor

After optimizing the conditions for the generation of alginate hollow fibers, enzyme-entrapped microfibers were generated. Ca-alginate formed by high or low gel-strength alginate permits diffusion of low molecular-weight (MW) molecules such as the reactant used in this paper. To prevent diffusion of reactants from hollow fibers, we embedded fibers of the desired length into the PDMS mold. The fabrication of a PDMS-embedded enzyme-entrapped hollow alginate microfiber bioreactor is shown in Fig. 4(a) and a photograph of the device is shown in Fig. 4(b). To fabricate a PDMS-embedded hollow-fiber device, a hole was first made in the side wall of a Petri dish, and PDMS prepolymer mixed with curing agent (10:1 weight ratio) was then poured into the dish and cured for 30 min at 70 °C. Because of the short curing time, PDMS was not completely cured and remained somewhat sticky. Next, enzyme-entrapped alginate hollow microfiber, inserted in an 18G needle on one side, was placed in a Petri dish and more PDMS prepolymer was poured on top of the microfibers. The device was then cured at 70 °C for another 1 h. When PDMS was completely cured, a small cut was made near the edge of the alginate microtube to form a sample collection reservoir.

C. Enzyme reaction

Many marine algae including those of the *Corallina* family are known to produce volatile halocarbons under the influence of environmental stress. The synthesis pathway involves the use of VBPO enzyme.²⁹ As with all haloperoxidases, VBPO catalyzes the addition of bromine or iodine into organic substrates, producing a range of organic halogenated products.^{28,32} These biohalogenation reactions use hydrogen peroxide (H₂O₂) produced within the cell, to oxidize bromide anions (Br⁻) to form highly reactive hypohalous acid intermediates such as hypobromous acid (HOBr) or bromonium cations (Br⁺).³² These species can become substrates for a number of

FIG. 5. Reaction showing transformation of Phenol Red to Bromophenol Blue catalyzed by vanadium bromoperoxidase.

reactions, including halogenation of nucleophilic organic compounds or react with excess H_2O_2 to yield singlet oxygen in the absence of suitable substrates.³³ Figure 5 shows the reaction scheme for the bromo-oxidation of Phenol Red (yellow in color) to the brominated derivative, Bromophenol Blue (blue in color) under physiological conditions (pH 6.5); this is the model reaction which has been employed to demonstrate bioreactivity in our alginate hollow fibers loaded with VBPO. The reaction is carried out in a buffered solution, as described in experimental section, to ensure that the change in color is a result of biocatalytic activity and not pH. The bromo-oxidation of Phenol Red to the brominated derivative, Bromophenol Blue, was performed within an alginate hollow microfiber reactor embedded in PDMS support material. The fiber used in the present work was 4–4.5 cm in length with an inner diameter of about 250 μm (outer diameter around 300 μm), as confirmed by confocal microscopy, and it was fabricated by a size control method developed in the earlier work.²⁰ For comparison, the reaction was also performed in a batch system under the same buffer conditions as described in experimental section. The bulk reaction took place in a 1-cm-long quartz cuvette at room temperature. As during the fabrication of the PDMS embedded device, the whole assembly, including enzyme embedded alginate hollow fiber, was heated at 70 °C for 1 h, the enzyme solution used for batch reaction was incubated at the same temperature (70 °C) for an hour and equilibrated to room temperature before use.

D. Thermal stability test

In order to investigate thermal stability of the enzyme under the above-mentioned fabrication conditions, free enzyme and the immobilized enzyme in the alginate matrix were subjected to various temperatures. When the free the enzyme was incubated at a temperature range of 25–80 °C for 1 h, the enzyme retained its activity and there was probable enhancement at 40 °C (100% conversion in 50 min) when compared to the enzyme activity at 25 °C (120 min). But at temperatures higher than 40 °C, about 2.4, 3.5, and 3.8 times decrease in the activity was observed at 60 °C (120 min), 70 °C (170 min), and 80 °C (190 min), respectively. The obtained findings were in accordance with the reported results, where VBPO is reportedly shown to exhibit remarkable thermostability, retaining appreciable activity at 60–70 °C.³⁴ Based on these results, the enzyme was encapsulated in the alginate microfiber and incubated at 60 and 80 °C for 1 h. For the reaction in an enzyme-entrapped hollow-fiber bioreactor, a mixture of 0.025 mM Phenol Red, 0.5 mM potassium bromide, and 0.25 mM hydrogen peroxide in 50 mM MES buffer, pH 6.5, was injected into the hollow fibers embedded in a PDMS matrix; various flow rates were employed. Products were collected and absorbance was measured at 590 nm; product yields at different flow rates are shown in Table I. It was observed that with an increase in flow rate or a reduction in residence time, there was a gradual decrease in the yield. At a flow rate of 0.1 $\mu\text{l}/\text{min}$, about 80% of the Phenol Red was transformed to Bromophenol Blue, which corresponds to a residence time of 22 min. In the batch system, a similar yield was obtained after a reaction time of ~ 108 min. Thus, the reaction kinetics was ~ 4.9 -fold faster in the microfiber bioreactor when compared to the batch system. An increase in the higher yield was expected considering the inherent advantages of

TABLE I. Comparative yields of Bromophenol Blue using enzyme-loaded alginate hollow microfiber with and without chitosan coating.

No chitosan coated		Chitosan coated	
Flow rate, $\mu\text{l}/\text{min}$ (Residence time, min) ^a	Yield ^b (%)	Flow rate, $\mu\text{l}/\text{min}$ (Residence time, min) ^b	Yield ^c (%)
0.10 (22.00)	80 $\pm$ 3.5	0.50 (3.92)	96.8 $\pm$ 2.8
0.25 (8.90)	49 $\pm$ 2.7	1.00 (1.96)	92.7 $\pm$ 1.5
0.50 (4.41)	28 $\pm$ 3.4	2.00 (0.98)	56.2 $\pm$ 1.1
1.00 (2.20)	09 $\pm$ 4.0		
Batch reaction (%): yield 100% in 170.00 min			

^aDimension of hollow microfiber: L 4.5 cm, ID 250 μm .^bDimension of hollow microfiber: L 4 cm, ID 250 μm .^cExperiment was repeated 03 times.

using a microreactor, as high mass-transfer rates possible in microreactor allow reactions to be performed under more aggressive conditions than can be achieved with conventional reactors. This can often lead to improved reaction yields or product conversion. The lower yield using this novel biomicroreaction can be explained using the reports on enzyme release from calcium alginate gel. Due to the open lattice structure of the matrix, alginate gels have a wide pore size distribution.³⁵ It has been reported that low molecular-weight molecules, such as glucose and α -lactalbumin, can diffuse in and out of matrix without restraint.³⁶ While higher-molecular-weight species, such as albumin, g-globulin, and vanadium, bromoperoxidase (>65 kDa) can diffuse out of matrix but cannot diffuse into the matrix.³⁷ This differential behavior of alginate matrix is developed during the cross-linking in presence of high-molecular-weight molecules; the resulting matrix will have fewer ionic cross-links and therefore will be more porous, hence more prone to leaching from the matrix. Different type of coatings such as with chitosan,³⁸ poly-(lysine)/alginate,^{39,40} and other materials have been used as barrier to stop or reduce diffusion of encapsulated macromolecules.^{41–46} Based on the above-mentioned reports, enzyme-entrapped alginate hollow fibers were treated with a 2% (w/v) solution of chitosan for 1 h followed by rinsing with de-ionized water to remove unbounded chitosan.

E. Evaluation of chitosan coating

To evaluate the effectiveness of coating, relatively large pore alginate hollow fibers were fabricated using a 500 and 365 μm glass capillary as embedded template by a method described elsewhere.⁴⁷ Green fluorescence protein (GFP, MW: 27 kDa) was used as a model molecule (which is relatively smaller to VBPO molecule (MW: >65 kDa)⁴⁸ to control leaching from the alginate matrix. As an initial experiment, an alginate hollow fiber without any coating was filled with GFP. The GFP molecules diffuse into the matrix without any delay [Fig. 6(a)]. To confirm the diffusion of GFP “into” the alginate matrix, internal walls of alginate hollow fiber were treated with 2% (w/v) solution of chitosan. This alginate tube was then filled with GFP solution and observed for 24 h. As shown in Fig. 6(b), chitosan coating does not allow GFP molecules to diffuse into the matrix. To evaluate the diffusion of small organic molecules such as phenol red (MW: 354.38 Da), a experiment similar to that mentioned for GFP diffusion was performed. Phenol Red diffuses into the bare alginate matrix as well as in the matrix coated chitosan readily without any delay [Fig. 6(c)]. Thus, from this these experiments, it was concluded that alginate matrix coated with chitosan would not allow VBPO to leach out from the alginate matrix.

After assuring the effectiveness of the chitosan coating, bromo-oxidation of phenol red to bromophenol blue was again performed in the chitosan coated enzyme-entrapped alginate fabricated by the microfluidic way. As expected, these results showed significant improvement with much faster reaction kinetics observed. As can be seen in Table I, nearly complete conversion (>96.8%) of phenol red was achieved within 3.9 min compare to >28% conversion in approxi-

FIG. 6. Effect of chitosan coating on alginate hollow microfiber. (a) A bare alginate hollow microfiber with diffused GFP. (b) Hollow microfiber with chitosan coating of inner wall showing no evidence of inwards diffusion of GFP; diffusion of phenol red in to the bare alginate matrix. [(c) and (d)] From alginate hollow fiber coated with chitosan.

mately a similar time frame from the alginate hollow fiber without chitosan coating. It was evident that more than 41-fold faster reaction conversion can be achieved by using chitosan coated enzyme entrapped alginate hollow fiber when compared with the bulk reaction time. These results also indicated that enzyme leaching can be controlled significantly by the chitosan coating. It was shown in a previous study that the reaction rate was faster in a microchannel than in a beaker.⁴⁹ Although the exact reason for the acceleration is not yet known, one of the possible and most accepted reason is an increase in surface to volume ratio in the microchannel, on which catalyst molecules may be adsorbed. Additionally, the porous nature of the alginate matrix further increases the surface to volume ratio for interaction of biocatalyst with reactants.

IV. CONCLUSION

In this article, for the first time, it has been demonstrated that a simple, single-step production of alginate hollow fibers loaded with enzyme can be employed using a microfluidic route. Additionally, the activity of the hollow fibers in the biocatalytic transformation of Phenol Red to a brominated derivative in the presence of VBPO has been successfully demonstrated. The reaction kinetics was 41-fold faster than in a batch process. Our results suggest that further exploration of enzyme-entrapped hollow tubes or fiber bioreactors is warranted for applications in biotransformation or chemical synthesis.

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