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On-Cell Surface Cross-Linking of Polymer Molecules by Horseradish Peroxidase Anchored to Cell Membrane for Individual Cell Encapsulation in Hydrogel Sheath

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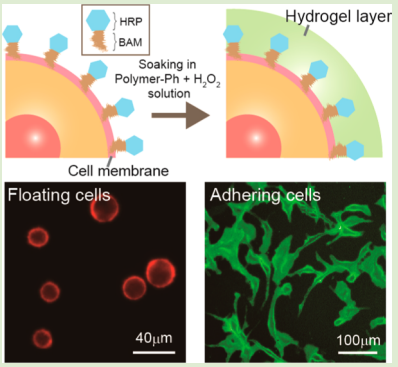
Supporting Information

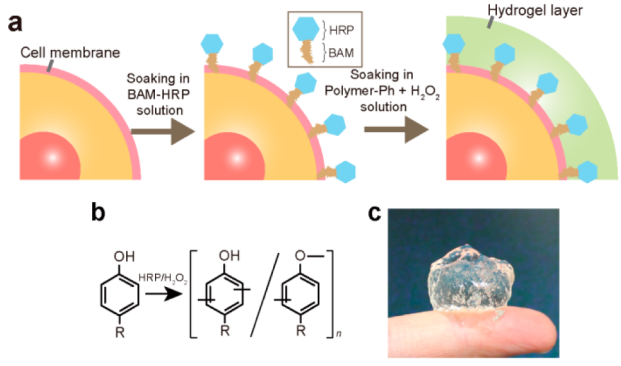
ABSTRACT: Hydrogel sheaths were fabricated on the surfaces of individual mammalian cells through the cross-linking of polymer molecules catalyzed by horseradish peroxidase (HRP) in aqueous solution. For confining the progress of the cross-linking only on the cell surface, HRP was anchored to the cell membrane by soaking the cells in the solution containing the HRP conjugated with a biocompatible anchor molecule for cell membrane. The hydrogel sheath of about 1 μm thickness was obtained by soaking the cells with the anchored HRP in aqueous solution containing polymers possessing phenolic hydroxyl (Ph) moieties and H_2O_2 for 10 min. The hydrogel sheaths could be made from a variety of polymers possessing Ph moieties, for example, derivatives of polysaccharide, protein, and synthetic polymer. Cytocompatibility of the on-cell surface enzymatic hydrogel sheath formation was confirmed from the viability of the enclosed cells (>90%) and subsequent normal growth after removal of the hydrogel sheath.



Cell encapsulation technology has advanced, increasing its application in a variety of fields including research tools for fundamental studies in cell biology, production of biomolecules, and cell therapies.[1−3] A recent direction for this technology has been the development of methods for encapsulating individual cells.[4−6] While a number of methods have been reported,[5] the challenge remains to develop a method that is cytocompatible with mammalian cells and that enables the sheath to be made from a variety of materials with different properties, such as electrostatic charge, cell adhesiveness, biodegradability, biocompatibility, and mechanical toughness. Such a method would be useful for fundamental studies on cellular metabolism at the single-cell level and cell-to-cell communications. In addition, it would increase the options available for implantation sites in cell therapy, and enable easy mechanical manipulation of cells. The difficulty in developing individual mammalian cell encapsulation methods is attributed to the inability of mammalian cells to withstand harsh conditions such as abnormal pH, temperature, ion concentrations, and toxic materials.[5] In addition, mammalian cell surfaces are not reinforced or protected by a layer of polysaccharide.[5] Therefore, careful selection of materials and processes are more necessary for encapsulating mammalian cells than for microorganisms. Layer-by-layer assembly of positively and negatively charged polymer molecules has been identified as a possible approach for individual mammalian cell encapsulation.[7,8] A drawback of this method is that only the polymers having suitable electrostatic charges are applicable.

Here, we propose a method that enables the encapsulation of individual mammalian cells within thin hydrogel sheaths made from a variety of polymers. The method uses a horseradish peroxidase (HRP)-catalyzed reaction for the formation of the hydrogel sheath. The enzyme is immobilized on the cell surface to confine the formation of hydrogel only on the surface of the individual cells (Scheme 1a). HRP catalyzes oxidative coupling of phenol derivatives in the presence of H_2O_2, yielding polyphenols linked at the aromatic ring by C−C and C−O

Scheme 1. (a) Hydrogel Sheath Formation on Cell Surface; (b) HRP-Catalyzed Cross-Linking of Ph Moieties on Polymer (R); and (c) Photograph of Flu−Alg−Ph Hydrogel Obtained through HRP-Catalyzed Reaction



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coupling (Scheme 1b). An attractive point of this enzymatic synthesis method is the abundance of candidates available as hydrogel sheath material. A required condition for the hydrogel sheath material is that it possesses moieties that are cross-linkable through the enzymatic reaction. Hydrogels have been prepared from aqueous solutions of a variety of polymers, for example, natural sugar beet pectin,[9] polysaccharide derivatives,[10−12] protein derivatives,[13,14] and synthetic polymers,[15,16] dissolving HRP as a homogeneous catalyst (Scheme 1c). To immobilize HRP onto the mammalian cell surface, we used a conjugate of HRP and a biocompatible anchor molecule (BAM) for cell membranes (BAM−HRP, Scheme 1a). BAM is a single oleyl chain derivative coupled with hydrophilic poly(ethylene glycol) (PEG).[17] The efficiency of BAM for anchoring a variety of functional molecules to mammalian cell surfaces has been described.[17]

To investigate the feasibility of our strategy, we first sequentially suspended mouse embryonic fibroblast cell line STO cells in a solution containing BAM−HRP (12 μg/mL) for 10 min. Then we soaked the cells in a solution containing a derivative of alginate possessing Ph and fluorescein moieties (Flu−Alg−Ph, 1.0% (w/v)) and H_2O_2 (0.1 mM) for 10 min (Supporting Information). Fluorescence attributed to the existence of Flu−Alg−Ph was detected on individual cells (Figure 1a). In contrast, the fluorescence was not detected on the surface of cells suspended in the Flu−Alg−Ph + H_2O_2 solution alone (Figure 1b). These results clearly demonstrate the feasibility of our strategy for individual mammalian cell encapsulation within a hydrogel sheath. The thickness of the sheath observed using a confocal fluorescence microscope was about 1 μm (Figure 1c). The method was also effective for hydrogel sheath formation on cells adhering to a substrate (Supporting Information). Spreading morphology was clearly visible using a fluorescence microscope through the formation of a Flu−Alg−Ph hydrogel sheath over the entire cell surface (Figure 1d).

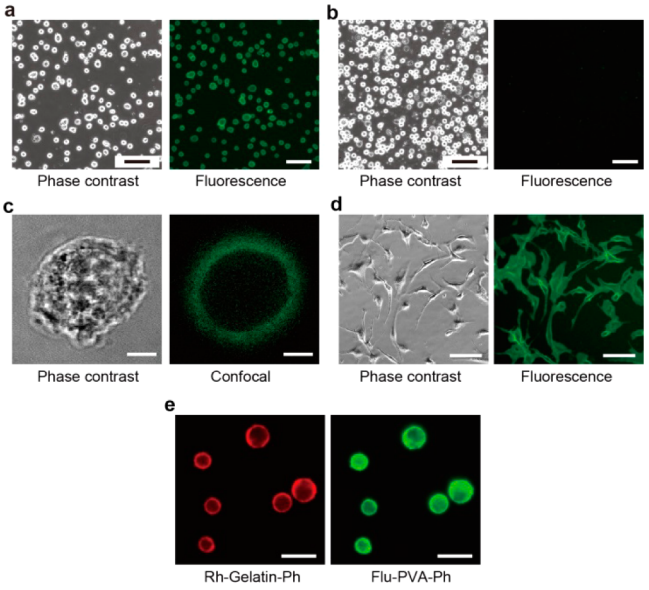

Figure 1. STO cells (a) sequentially treated with BAM-HRP solution and Flu−Alg−Ph + H_2O_2 solution after trypsinization, (b) treated with Flu−Alg−Ph + H_2O_2 solution alone without BAM-HRP solution treatment after trypsinization, (c) phase contrast and confocal fluorescence images of the cell treated with the same conditions shown in (a), and (d) treated sequentially with BAM-HRP solution and Flu−Alg−Ph + H_2O_2 solution without being detached from culture dish. (e) HeLa cells sequentially treated with BAM-HRP solution and Rh−gelatin−Ph (red fluorescence) + Flu−PVA−Ph (green fluorescence) + H_2O_2 solution. Bars in (a), (b), (d): 100 μm; (c): 2 μm, and (e): 40 μm.

The versatility of the method was confirmed by hydrogel sheath formation on human hepatoma HepG2 cells, human cervical cancer HeLa cells, and human epithelial cells in solutions containing a derivative of various biocompatible polymer possessing incorporated fluorescent and Ph moieties, hyaluronic acid (polysaccharide), poly(vinyl alcohol) (Flu−PVA−Ph, synthetic polymer), or gelatin (Rh−gelatin−Ph, protein) with H_2O_2 (Supporting Information, Figure S2). Additionally, a hydrogel sheath could be formed in a solution containing multiple polymers possessing Ph moieties, Rh−gelatin−Ph and Flu−PVA−Ph, with H_2O_2 (Figure 1e). Hydrogel sheaths formed from polysaccharide derivatives are useful from the viewpoint of removing the sheaths, on demand, using nonproteolytic degradation enzymes. It is known that proteolytic degradation enzymes digest membrane-proteins.[18] The treatment with polysaccharide degradation enzymes is harmless to mammalian cells.[18−20] Potential applications of the encapsulation of cells in the on-demand degradable hydrogel sheaths are the studies of the effects of stimuli given to the physically isolated cells on the subsequent behaviors such as growth. A sheath derived from a poly(vinyl alcohol) derivative is attractive from the viewpoint of preparing hydrogel sheaths with a high durability against deformation.[21] Including the sheaths derived from the polysaccharide and poly(vinyl alcohol) derivatives, the sheaths nondegradable with proteolytic enzymes in vivo would be useful in cell therapies for isolating cells from the host immune system.[1] A sheath comprising a gelatin derivative would be useful for developing artificial tissues through structural assembly. Further, the possibility of preparing a sheath comprising multiple polymers conjugated by the HRP-catalyzed reaction is attractive for obtaining customizable sheaths with specified properties tuned for individual applications. It is well-recognized that hydrogels composed of multiple polymers show unique properties that cannot be obtained using either of the components alone.[22,23]

We next investigated the possibility of tuning the content of polymer molecules within the hydrogel sheath. Intuitively, the polymeric makeup of the hydrogel sheath influences properties such as the mechanical characteristics, permeability and electrostatic charge. These characteristics would govern the behaviors of the encapsulated cells. Regulation of cell behaviors by these factors has been intensively investigated.[24−26] We studied the effects of soaking time in Flu−Alg−Ph and concentrations of BAM−HRP, Flu−Alg−Ph, and H_2O_2 by measuring the fluorescent intensity attributed to Flu−Alg−Ph, using a flow cytometer. Fluorescent intensities increased with soaking time in Flu−Alg−Ph solution, and with concentrations of Flu−Alg−Ph and BAM−HRP (Figure 2a−c). These results are consistent with the general principle of enzyme kinetics with no inhibition. Regarding the effect of H_2O_2, the Flu−Alg−Ph content increased with H_2O_2 concentration from 0.1 to 1.0 mM. However, further increase to 10 mM decreased the fluorescent intensity (Figure 2d). This result may be attributed to the deactivation of HRP by H_2O_2, inhibiting the progress of HRP-catalyzed reaction. Similar results have been reported in the hydrogelations of whole Alg−Ph solution[27] and other polymer−Ph solutions[10,12] using nonimmobilized HRP. In

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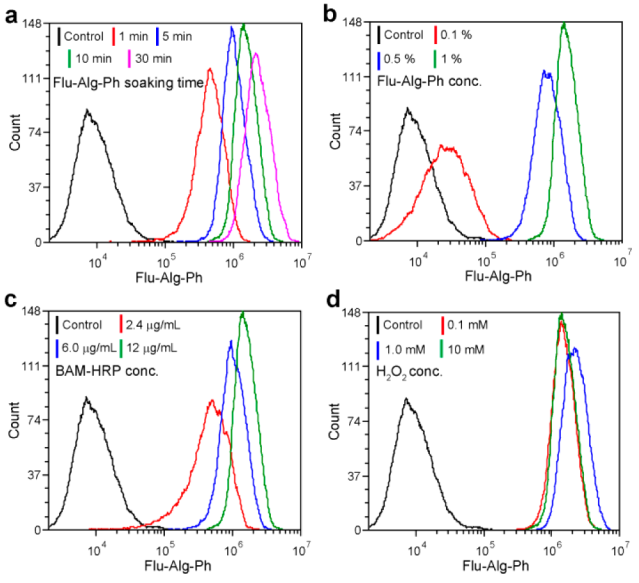

Figure 2. Effects of (a) soaking time in 1.0% (w/v) Flu−Alg−Ph + 0.1 mM H_2O_2 solution, (b) Flu−Alg−Ph concentration in 0.1 mM H_2O_2 solution, (c) BAM-HRP concentration, and (d) H_2O_2 concentration in 1.0% (w/v) Flu−Alg−Ph solution on fluorescent intensity attributed to existence of Flu−Alg−Ph on cell surfaces. The cells in (a), (b), and (d) were soaked in 12 μg/mL BAM-HRP solution for 10 min before soaking in each Flu−Alg−Ph + H_2O_2 solution. The cells in panels (b)−(d) were soaked in 1.0% (w/v) Flu−Alg−Ph + 0.1 mM H_2O_2 solution for 10 min after the treatment with BAM-HRP solution. Control was cells treated with Flu−Alg−Ph + H_2O_2 solution alone.

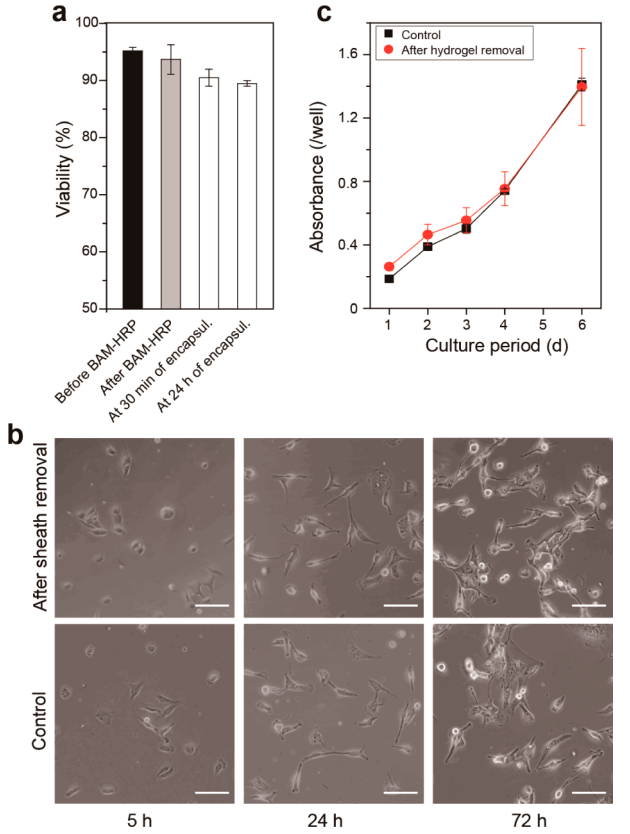

Figure 3. (a) Viabilities of STO cells before (black column) and after (gray column) BAM-HRP treatment, and at 30 min and 24 h of treatment with Flu−Alg−Ph + H_2O_2 (white column). Bars: SD. (b) Micrographs of STO cells grown on a cell culture dish after removal of hydrogel sheath by treatment with alginate lyase (after sheath removal) and seeded without being exposed to the treatment for hydrogel sheath formation (Control). Bars: 100 μm. (c) Growth profiles of cells after the removal of hydrogel sheath (red) and seeded without being exposed to the treatment for hydrogel sheath formation (control). The cells were seeded at the same density. Bars: SD.

previous reports, the time required for hydrogelation increased with increasing H_2O_2 concentration. The increase in fluorescent intensity when increasing the H_2O_2 concentration from 0.1 to 1.0 mM would arise from an increased HRP-catalyzed reaction rate through the increase in electron donor availability more than offsetting the associated decrease in reaction rate caused through enzyme deactivation by H_2O_2.

Finally, we studied the cytocompatibility of the encapsulation method by measuring the viabilities of the encapsulated cells by Trypan blue exclusion test. There were no observable adverse effects inducing a decrease in viability specific to the current method. The viability after 30 min of encapsulation was 90.5 ± 1.3% (n = 5; Figure 3a). This value is comparable to viability values reported for cells enclosed in hydrogels obtained through the hydrogelation of the whole polymer solution by homogeneously dissolved HRP.[13] Following 24 h of culture in a hydrogel sheath coating, there was no decrease in viability (89.5 ± 0.4% at 24 h of encapsulation, $p > 0.05$, vs that at 30 min of encapsulation). Additionally, we evaluated the possible occurrence of harmful effects that require time to induce cell death and growth inhibition by measuring the growth of cells after removal of the hydrogel sheath using alginate lyase. No significant differences were found in the morphology and growth profile of the cells obtained in this way compared with those of control cells, free from encapsulation (Figure 3b,c). In addition to the main focus of this study, that is, developing a novel method for single cell encapsulation in a hydrogel sheath, on-demand degradability of Flu−Alg−Ph hydrogel sheaths by treatment with alginate lyase would be useful in a variety of applications, for example, studies in single-cell biology after a certain period of physical isolation from an external environment.[5]

In summary, we report a cytocompatible method for individual mammalian cell encapsulation in a thin hydrogel sheath. The hydrogel sheath formation is mediated by HRP immobilized on the cell surface by BAM conjugated with the enzyme. The HRP anchored to the cell surface then catalyzes the cross-linking of Ph moieties in polymer molecules from the surrounding aqueous solution, and results in the formation of the hydrogel sheath at physiological pH and temperature. It is worthy of note that no significant adverse effects of the encapsulation method on viability and subsequent proliferation of encapsulated mouse fibroblast cells were observed. Additionally, because the hydrogel sheath can be prepared from a variety of polymers possessing Ph moieties, the method proposed here is expected to be useful for a wide range of applications and will extend the applications of cell encapsulation technology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and additional data of BAM-HRP preparation, synthesis of Flu-Alg-Ph, hydrogel sheath formation, and versatility in materials. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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