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Development of Double-Layered Alginate-Chitosan Hydrogels for Human Stem Cell Microencapsulation

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Abstract. Microencapsulation is a promising technology for stem cell therapy and tissue regeneration. Our previous work proposed the application of microencapsulation technology for improving allogeneic hematopoietic stem cell transplantation. In this current work, we developed a feasible method for encapsulating human umbilical cord blood-derived hematopoietic stem cells. The cells were initially entrapped in ionic cross-linked alginate beads based on a conventional method. It was noticed that alginate beads were easily dissolved in phosphate-buffered saline with Ca2+/Mg2+, suggesting a poor stability of alginate beads. Therefore, a double-layered microcapsule was developed by coating the alginate gel with glutaraldehyde cross-linked chitosan. Measurement of relative swelling ratio showed that the double-layered gel could expand 1.49-fold in a rich culture medium. A homogenous cell distribution could be visualized in solid core alginate microcapsule by a DNA staining. Altogether, this study presented a feasible method to fabricate a double-layered alginate-chitosan microcapsule for human stem cells.

Keywords: alginate, chitosan, hydrogel, microencapsulation, stem cells,

INTRODUCTION

Encapsulation is a process to immobilize and envelop the inner substance with a polymer layer. In regenerative medicine, encapsulation technology is used to reduce immune rejection for allogeneic transplantation and to control the release of bioactive compounds. The polymer for cell encapsulation requires certain characteristics, i.e. biodegradable, biocompatible, non-toxic, stable in the desired environment, and permeable for nutrient-metabolite
exchange. The most utilized biomaterials for cell encapsulation are collagen, alginate, gelatin, and chitosan [1-2]. The bead size is an important parameter for cell encapsulation. Bigger bead size will decrease the nutrient/gas exchange. Thus, micro-sized bead for microencapsulation is often desired to get the benefit of encapsulation without harming the entrapped cells. In this current study, we developed a feasible microencapsulation method with alginate and chitosan materials.

Alginate is a natural and hydrophilic polysaccharide from marine algae. Alginate gel can be easily formed in the presence of multivalent ion, i.e. Ca\(^{2+}\), Ba\(^{2+}\), Fe\(^{3+}\) [3]. The gelation process of alginate can be performed in single step under the very gentle condition without compromising cell viability [4]. Therefore, alginate-based hydrogel is still the most popular material for cell encapsulation. However, alginate gel can be destabilized by chelating agents and non-gelling ion, i.e. Mg\(^{2+}\) [4]. Therefore, we used glutaraldehyde cross-linked chitosan as a coating layer for improving the microcapsule stability. Chitosan is a biodegradable and biocompatible material. Even though not readily available in nature, it can be easily synthesized from partial deacetylation of natural chitin polymers. The gelation process of chitosan requires harsh condition which potentially harmful for human cells. Nevertheless, it is relatively stable and has low degradability in various condition.

Hematopoietic stem cells (HSCs) are multipotent stem cells which can differentiate into various types of blood cells [5]. HSCs can be obtained from several sources, i.e., bone marrow isolation, umbilical cord blood, and peripheral blood [6]. In the human body, HSCs are mainly stored in the bone marrow and classified as rare cells since the availability is less than 0.1% of all nucleated bone marrow cells [7]. The multipotent nature of HSCs is often used to cure degenerative and blood-related terminal diseases such as thalassemia, leukemia myeloma, or autoimmune diseases through cell transplantation. However, there is rejection risk from the immune system when transplanted. Therefore, microencapsulation technology is needed to reduce HSCs transplantation failure due to immune system rejection [8]. Our previous work proposed the application of encapsulation technology for improving allogeneic HSCs transplantation [9]. In this current work, we developed a novel method for fabricating double-layered alginate-chitosan microcapsules containing human umbilical cord blood-derived hematopoietic stem cells.

**MATERIAL AND METHODS**

**HSCs Isolation**

Umbilical cord blood was collected from Dr. Cipto Mangunkusumo General Hospital after the participants were given informed consent. The protocols used in this study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo General Hospital. HSCs isolation was conducted as described in our previous work [9]. Briefly, HSCs were purified from umbilical cord blood by a magnetic-sorting method. Cell viability was counted by a dye exclusion method using trypan blue.

**Hydrogel Formation**

**Alginate Microcapsules**

Sodium alginate (Sigma-Aldrich, USA) was dissolved using phosphate-buffered saline without Ca\(^{2+}\) and Mg\(^{2+}\) (PBS w/o Ca/Mg) (Sigma-Aldrich) until a final concentration of 50 mg/ml. Alginate solution was mixed with cell suspension (5 × 10\(^6\) cells/ml) in ratio 4:1. The mixture (10 \(\mu\)l) was dropped into 0.2 M CaCl\(_2\) solution under agitation at 230 rpm. The alginate microcapsules were subsequently washed with PBS w/o Ca/Mg.

**Double-layered Alginate-chitosan Microcapsules**

Alginate microcapsules were immersed in 1% glutaraldehyde solution and transferred into 10 mg/ml chitosan solution (Sigma-Aldrich, USA). The chitosan coating was neutralized with 0.1 M NaOH solution. The double-layered gel washed with PBS w/o Ca/Mg.
Swelling Test

Double-layered alginate-chitosan microcapsules (three gels per group) were weighed before and after immersion with an analytical balance (Kern, Germany). Five groups of microcapsules were immersed in PBS w/o Ca/Mg and the non-absorbed water was removed from gels with a cellulose tissue (Fisher Scientific, USA) prior to weighing. The measurements were done every hour until the weigh reached the plateau.

Cell Morphology Analysis

The microcapsules were immersed in paraformaldehyde (Sigma-Aldrich, USA) for 15 min to permeabilize the cells. The gels were washed twice with PBS w/o Ca/Mg and immersed in cold methanol for an hour. After 30 min treatment with RNAse (Sigma-Aldrich, USA), the gels were stained with propidium iodide (Wako Pure Chemicals, Japan) for 15 min. The stained gels were observed under a fluorescence microscope (Zeiss, Germany).

Statistical Analysis

Data were presented as mean values ± standard deviations. Data sets were analyzed using one-way ANOVA, with post-hoc analysis using the paired t-test. The value of $p<0.05$ was considered as a significant difference.

RESULTS AND DISCUSSION

Alginate microcapsules were successfully produced by a conventional method. However, it was noticed that alginate microcapsules were easily degraded when immersed in PBS with Ca/Mg (Figure 1). Freshly fabricated capsules had a rounded shape with a good integrity. After three days of incubation in PBS with Ca/Mg, the capsules started to deteriorate and on day 5, the gels were completely degraded. The stability of alginate gel is highly compromised by ion and chelating agent since alginate can be cross-linked by divalent cations except for Mg$^{2+}$ [4]. Accordingly, we coated the alginate beads with glutaraldehyde cross-linked chitosan.

FIGURE 1. The stability of alginate microcapsules on (a) day-0, (b) day-3, and (c) day-5 in PBS (with Ca and Mg). Black arrows indicate microcapsule border. Scale bars indicate 200 µm.
The double-layered alginate-chitosan microencapsulation was successfully fabricated (Figure 2) by two-steps process. The double-layered microcapsules had round shape and diameter ~ 1,000 µm. Alginate has the advantage of being a biodegradable and biocompatible compound [3]. The gentle gelation method and non-toxic characteristics make the alginate polymer is a suitable material for cellular encapsulation [10]. Meanwhile, chitosan needs to be dissolved in acidic solution which makes the direct cellular microencapsulation with chitosan potentially reduce the cell viability.

![Figure 2](image)

**FIGURE 2.** The representative image of human umbilical cord blood-derived cells entrapped in a double-layered alginate-chitosan microcapsule. Scale bar indicates 200 µm.

To distinguish the entrapped cells with empty bubbles or debris, the microcapsules were stained with propidium iodide (PI), a nucleic acid dye. PI specifically binds to the cell nucleus; therefore, only cells will emit red light under a fluorescence microscope [11]. In this study, the microcapsules were treated with paraformaldehyde and methanol prior staining process to permeabilize the cell membrane. Figure 3 showed clear evidence that the cells were homogeneously distributed in the double-layered microcapsules.

![Figure 3](image)

**FIGURE 3.** (a) Bright-field microscopic image of encapsulated cells. (b) Fluorescence microscopic images of encapsulated cells. Scale bars indicate 200 µm.

Swelling rate of double-layered microcapsules was analyzed to know the substrate/water exchange in or out beads (substrate/water uptake) [12]. The results showed that the swelling rate increased significantly in the first hour and
started to stable in the next hour (Figure 4). An hour after immersion in the culture medium, the beads were swollen up to 1.49-fold. This results suggested that the double-layered microcapsules were permeable enough to allow substrate/water exchange.

CONCLUSION

Single-layered alginate beads were easily dissolved in phosphate-buffered saline with Ca\(^{2+}/\)Mg\(^{2+}\), suggesting a poor stability of alginate beads. Therefore, a double-layered microcapsule was developed by coating the alginate gel with glutaraldehyde cross-linked chitosan. Measurement of relative swelling ratio showed that the double-layered gel could expand 1.49-fold in a rich culture medium. A homogenous cell distribution could be visualized in solid core alginate microcapsule by a DNA staining. Altogether, this study presented a feasible method to fabricate a double-layered alginate-chitosan microcapsule for human stem cells.

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