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Alginate-Chitosan Microencapsulated Cells for Improving CD34$^+$ Progenitor Maintenance and Expansion

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Abstract: Protocols for isolation, characterization, and transplantation of hematopoietic stem cells (HSCs) have been well established. However, difficulty in finding human leucocyte antigens (HLA)-matched donors and scarcity of HSCs are still the major obstacles of allogeneic transplantation. In this study, we developed a double-layered microcapsule to deliver paracrine factors from non-matched or low-matched HSCs to other cells. The umbilical cord blood-derived hematopoietic progenitor cells, identified as CD34$^+$ cells, were entrapped in alginate polymer and further protected by chitosan coating. The microcapsules showed no toxicity for surrounding CD34$^+$ cells. When CD34$^+$ cells-loaded microcapsules were co-cultured with bare CD34$^+$ cells that have been collected from unrelated donors, the microcapsules affected surrounding cells and increased the percentage of CD34$^+$ cell population. This study is the first to report the potency of alginate-chitosan microcapsules containing non-HLA-matched cells for improving proliferation and progenitor maintenance of CD34$^+$ cells.

Keywords: hematopoietic; CD34; progenitor; stem cells; microencapsulation; chitosan; alginate; proliferation; megakaryocyte

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1. Introduction

Hematopoietic stem cells (HSCs) are multipotent cells capable of generating all blood components, including erythrocytes, leucocytes, and thrombocytes [1]. HSCs represent only a fraction of the cell population in umbilical cord blood, bone marrow, or peripheral blood. To isolate the HSCs from heterogenous populations, specific surface markers such as HLA-DR, CD38, CD117 (c-kit), CD45, CD133, and/or CD34 have been used [2]. One of the prominent surface markers that is widely used to isolate hematopoietic progenitor

from heterogenous populations is CD34 [3,4]. $CD34^+$ cells that have enhanced progenitor activity represent a small proportion of the population [4]. Previous studies have reported that transplantation of $CD34^+$ cells successfully established multi-lineage hematopoietic engraftment [5] and improved neurobehavior of animals with stroke [6,7], brain injury [8], and cerebral palsy [9].

In clinical cases, HSCs transplantation is required for various applications, including repeated chemo- or radiotherapy for cancer patients, leukemia, lymphoma, or blood and bone marrow disorders [10]. Although the efficacy has been proven and protocol has been established, providing HLA-matched HSCs is challenging due to the diversity of HLA types and because the cells hardly propagate ex vivo.

In this study, we propose microencapsulation of non- or low-matched $CD34^+$ cells for protection against immune rejection and facilitating paracrine excretion. Microencapsulated $CD34^+$ cells can be utilized to deliver paracrine factors of unrelated $CD34^+$ cells for ex vivo expansion with a bioreactor system. For a long-term target, the microencapsulation system can be further developed to deliver paracrine factors, which provide signals to resident HSCs for regulating the homeostasis of HSCs in their niche. Two main activities that should be performed by HSCs to maintain homeostasis are cell proliferation and stemness preservation.

The novelty of this study lies in the design of a 3-dimensional (3D) scaffold for encapsulating $CD34^+$ cells and the idea for co-cultures between $CD34^+$ cells from different donors. To our knowledge, published works on 3D HSC scaffolds were generally used for ex vivo HSC culture and expansion systems and as a model for interaction between HSCs and their microenvironment [11,12]. Alginate-based capsules [13,14], 3D polyethylene terephthalate-based nanofiber [15], and collagen gel [16] have been studied as mesenchymal stem cells (MSCs) loaded scaffolds [17] and HSC-MSC micro-aggregates [16]. In these studies, biocompatibility, durability, and transplant feasibility have not always been the main considerations for designing the systems. We initially developed collagen-based scaffolds for encapsulating $CD34^+$ cells [18]. Cell viability was not affected by the collagen-based encapsulation, but it is likely that collagen promotes cell differentiation as indicated by decreasing $CD34^+$ cell population [18]. In the next step, alginate-based microcapsules were constructed [19]. In this paper, chitosan, a biocompatible polymer [20], was cross-linked with glutaraldehyde [21] and used as an outer layer to further improve alginate microcapsule stability [22]. The impact of co-culture between microencapsulated $CD34^+$ cells and bare $CD34^+$ cells from different donors was investigated. Paracrine effects by the microencapsulated $CD34^+$ cells towards the bare $CD34^+$ cells in the lower well were analyzed for cell viability and progenitor maintenance represented by the CD34 marker.

2. Materials and Methods

2.1. $CD34^+$ Cell Isolation

$CD34^+$ cell isolation from unrelated donors was conducted as described in our previous work [18]. In brief, mononuclear cells were isolated from human umbilical cord blood (UCB) by a density gradient technique with Lympoprep (STEMCELL Technologies, Vancouver, CO, Canada). UCB was collected from Dr. Cipto Mangunkusumo General Hospital after the participants were given informed consent. Ethical clearance for this study was ratified by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia—Dr. Cipto Mangunkusumo General Hospital. $CD34^+$ cells were purified from mononuclear cells by EasySep™ Human Cord Blood CD34 Positive Selection Kit II (class II anti-CD34 antibody clone; STEMCELL Technologies, Vancouver, CO, Canada).

2.2. Alginate-Chitosan Microcapsule Fabrication

Microcapsules were fabricated based on our previous work [19]. Briefly, 1 part cell suspension (5×10^6 cells/mL) was mixed with 4 parts alginate solution (50 mg/mL) and then the mixture was dropped (10 µL/drop) into $CaCl_2$ solution. The alginate microcapsules were subsequently coated with glutaraldehyde cross-linked-chitosan (10 mg/mL)

and neutralized with NaOH (1N). Microcapsules were washed 3 times with Mg/Ca-free phosphate-buffered saline (PBS) and tested in phenol red medium to assure neutral acidity. All reagents were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. DNA staining and microscopic observation of microencapsulated cells were performed according to our published work [18].

2.3. Cytotoxicity Assay

In each experiment, 5 alginate-chitosan microcapsules were soaked in 300 µL phenol red-free RPMI medium supplemented with 10% outdated platelet lysate (Indonesian Red Cross (PMI), Jakarta, Indonesia), heparin (10 U/mL) (Fahrenheit, Indonesia), and antibiotic-antimycotic (Life Technologies, Carlsbad, CA, USA) for 72 h. The media was filtered to remove the microcapsules and subsequently used to culture $CD34^+$ cells in 96 well-plate (10^4 cells/well) (Biologix, Changzhou, China). Control cells were $CD34^+$ cells cultured in fresh media. After 24 h incubation, an equal volume of MTT solution (ThermoFisher, Waltham, MA, USA) was added directly to the culture and incubated for an additional 24 h at a refrigerated shaker (~4 °C). Insoluble formazan was dissolved with 25 µL dimethyl sulfoxide (DMSO), and the absorbance was measured at 540 nm with a spectrophotometer (Varioskan, ThermoFisher, Waltham, MA, USA).

2.4. Cell Leakage Detection Assay

Cell-loaded microcapsules were incubated in platelet lysate-supplemented RPMI media (6 microcapsules per mL media) and then incubated at 37 °C, 5% CO_2 in a fully humidified incubator. On day 8, the microcapsules were removed, and the media were collected. The detection of cell leakage from microcapsules was conducted by microscopic observation and DNA analysis of soaked media. DNA isolation was conducted according to a manufacturer's protocol (Geneaid, Taiwan) and DNA concentration was measured at 260 nm with a spectrophotometer (Biochrom, Cambridge, UK). Non-encapsulated cells (10^6 cells/mL) and fresh media were used as positive and negative controls of DNA detection, respectively.

2.5. Co-Cultures of Microencapsulated $CD34^+$ Cells with Bare $CD34^+$ Cells

$CD34^+$ cells in various concentrations (2.5, 5, or 10×10^4 cells/mL) were cultured in a 24 multiwell plate containing 500 µL StemSpan medium (Stem Cell Technologies) supplemented with thrombopoietin (10 ng/mL; Wako Pure Chemical, Osaka, Japan), 10% outdated platelet lysate, heparin, and antibiotic-antimycotic. In the 1st experiment set, 3 $CD34^+$ cell-loaded microcapsules were placed in each well and separated from bare cells by a polycarbonate cell culture insert (ThermoFisher, Waltham, MA, USA). In the 2nd experiment set, seeded cells (2.5×10^4 cells/mL) were co-cultured with 3×10^4 cells/well of non-encapsulated (bare) or encapsulated cells. The $CD34^+$ cells for encapsulated and non-encapsulated (bare) cells in the upper well and bare cells in the lower well from 1st and 2nd experiment sets were collected from unrelated donors. Half of the media was changed after 4 days, and the cells were harvested on day 8. Viable cell counting was performed by a dye exclusion method using trypan blue (Life Technologies, Carlsbad, CA, USA).

2.6. Flow Cytometry Analysis

Flow cytometry analysis was performed as described elsewhere [18]. In brief, the cells were stained with PE-conjugated CD34 (class III antibody clones 8G12) or FITC-conjugated CD41 antibodies (BD Biosciences, San Jose, CA, USA) for 30 min at 4 °C. The stained cells were analyzed with FACSAria III flow cytometer (BD Biosciences, San Jose, CA, USA).

2.7. Statistical Analysis

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

3. Results and Discussion

3.1. Fabrication of a Durable Double-Layered Microcapsule

A double-layered alginate-chitosan microcapsule was fabricated to encapsulate $CD34^+$ progenitor cells (Figure 1A,B). The coating is intended to protect the cells from immune cell intrusion and, at the same time, to be permeable enough to allow nutrient and gas exchange or paracrine secretion (Figure 1A). The core is alginate-entrapped cells, and the outer layer consists of chitosan cross-linked with glutaraldehyde (Figure 1B,D). The glutaraldehyde cross-linked chitosan coating improved alginate microcapsule stability (Figure 1C). Based on microscopic observations, the alginate-chitosan microcapsules retained its spherical shape for at least 12 days in culture media. The DNA analysis was conducted to confirm no cell leakage from microcapsules into media. Non-encapsulated cells and fresh media were used as positive and negative controls of DNA detection, respectively. DNA concentration was zero for microcapsule-soaked media (n = 3) and fresh media, whereas DNA concentration of positive control (10^6 cells) was 26.3 ng/µL. Chelating agents (i.e., phosphate, citrate, lactate) and non-gelling cations (i.e., Na^+, Mg^{2+}) contained in the media are known to destabilize calcium alginate gel [23]. Non-coated calcium alginate microcapsules typically break down after 3-day immersion in common saline [19]. Our experimental data suggested the alginate-chitosan microcapsules were able to provide a physical barrier in the employed culture condition.

Next, the cytotoxicity effect of alginate-chitosan microcapsules was analyzed by an indirect MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay [24] after microcapsules were immersed in media for 3 days. There was no significant difference of cell viabilities cultured in media with or without microcapsules (Figure 1E). This result suggests that the microcapsules do not pose a cytotoxic effect on surrounding cells.

3.2. Paracrine Effects of Microencapsulated $CD34^+$ Cells

To reconstitute the communication condition in HSC niche, co-culture experiments of microencapsulated $CD34^+$ cells and bare $CD34^+$ cells were set up. The experiments were performed in a static bath culture to facilitate paracrine factors excreted from microencapsulated cells. The microcapsules were placed on the top part, while the non-encapsulated/bare $CD34^+$ cells were in the lower part. A polycarbonate insert was used for easy cell harvest (Figure 2A). Initially, we prepared 3 microcapsules/well that contains a total of 6×10^3 cells/well (Donor 1; upper well) for co-culture with 2.5, 5, or 10×10^4 cells/mL (Donor 2, lower well). There was a significant increase in $CD34^+$ cell population of 2.5×10^4 cells (lower well) after 8-day co-culturing, in comparison with those without microencapsulated cells (data not shown). However, the $CD34^+$ ratio did not improve in higher seeding density cultures. With the same treatment, no impact was seen in cell proliferation of all seeding densities (Figure S1). Therefore, we increased the cell loading capacity of each of 3 microcapsules to contain 10^4 cells/microcapsule (total 3×10^4 cells/well) (Donor 3; upper well) for co-culture with 2.5, 5, and 10×10^4 cells/mL (Donor 4; lower well).

As seen in Figure 2B, microcapsules containing 3×10^4 cells per well improved the viable cell densities of bare $CD34^+$ cells (lower well) seeded in 2.5 and 5×10^4 cells/mL, but made no difference at a higher seeding density. At seeding density of 2.5×10^4 cells/mL and 5×10^4 cells/mL, cell concentrations of those with microencapsulated cells increased from $2.7 \pm 0.3 \times 10^4$ cells/mL to $4.7 \pm 0.6 \times 10^4$ cells/mL and from $3.5 \pm 0.5 \times 10^4$ cells/mL to $7.1 \pm 1.6 \times 10^4$ cells/mL, respectively. We expected that for seeding density 10×10^4 cells/mL required a higher dosage of microencapsulated cells.

Typically, freshly isolated $CD34^+$ cells from UCB have a purity of $93.0 \pm 0.2\%$ (indicated as a striped line in Figure 2C). After 8-day culture, the CD34 positive cell percentage dropped to 83.2 ± 1.7, 82.7 ± 2.0 and $88.4 \pm 0.5\%$ in $CD34^+$ cells seeded at 2.5, 5, 10×10^4 cells/mL, respectively. Interestingly, $CD34^+$ ratio increased to 89.3 ± 1.2, 96.0 ± 0.1 and $94.4 \pm 0.4\%$ in cultures treated with microencapsulated cells. These re-

sults suggested that microencapsulated cells potentially maintained CD34 expression in in vitro culture.

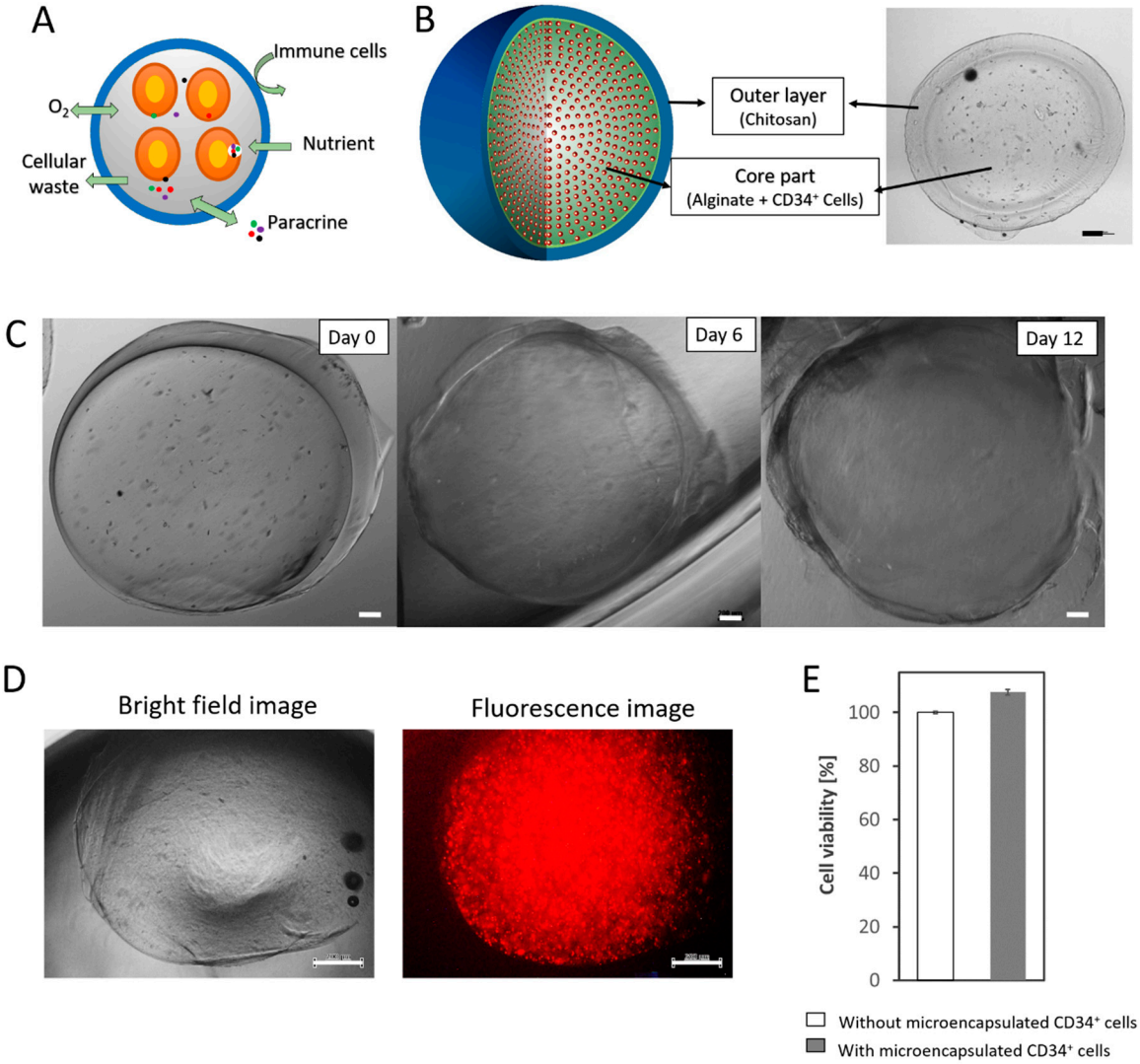

Figure 1. (**A**) Illustration of microencapsulation with a semi-permeable barrier. (**B**) Schematic and microscopic image of double-layered alginate-chitosan microcapsule. (**C**) Stability of alginate-chitosan microcapsules in culture media. (**D**) Microscopic images of cell-loaded microcapsule after DNA staining. Scale bars in (**B**–**D**) represent 100 and 200 μm, respectively. (**E**) Cytotoxicity of alginate-chitosan microcapsules for external $CD34^+$ cells.

3.3. Importance of Microenvironment for $CD34^+$ Progenitor Maintenance

Hematopoiesis, including self-renewal and differentiation, is highly regulated by the bone marrow microenvironment. Autocrine/paracrine factors such as growth factors, cytokines, and chemokines are secreted by early and differentiated hematopoietic cells, may play important roles as chemo-attractants for other hematopoietic cells (accessory cells, facilitating cells, etc.), stimulate the secretion of other regulatory molecules, modulate the expression of adhesion molecules on the cell surface (e.g., adhesion and homing), and/or influence the survival of the hematopoietic cells [25].

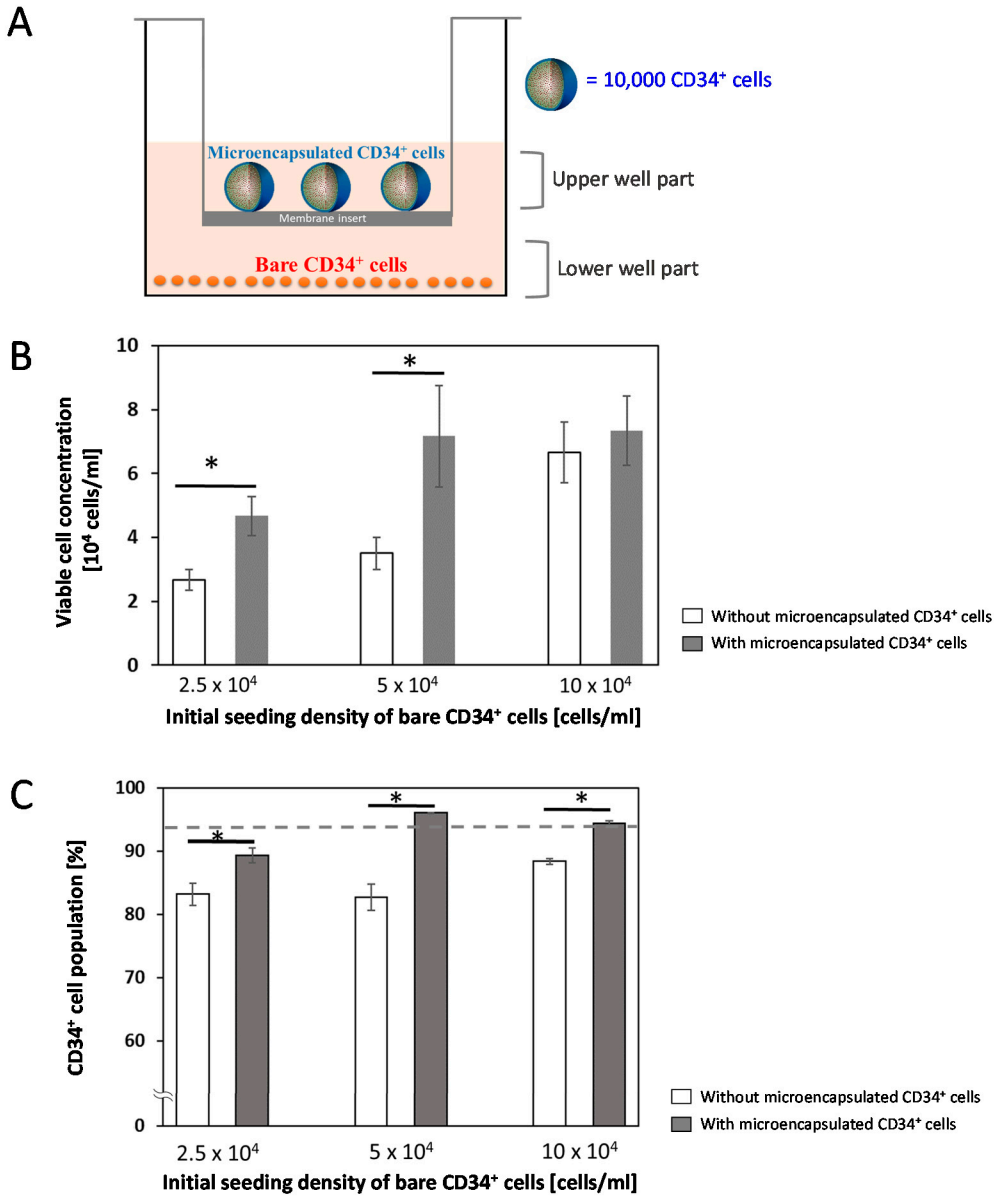

Figure 2. (**A**) Illustration of co-culture system of $CD34^+$ cell-loaded microcapsules (3×10^4 cells/well) with bare $CD34^+$ cells from unrelated donors. (**B**) Viable cell concentrations of bare cells (lower well) on day 8. (**C**) Ratio of $CD34^+$ cell population (lower well) on day 8. Striped line in C indicates percentage of $CD34^+$ cell population on day 0. Asterisks (*) in (**B**,**C**) indicate $p < 0.05$.

In a recent study, a micro-cavity platform was developed for isolating HSC to grow solely or in certain cell numbers thus that it can be used to investigate the impact of autocrine and paracrine signals in HSC culture [26]. The hypothesis was autocrine signals are predominantly involved in maintaining the quiescent state of HSC in single-cell niches [26]. However, it was implied that single-cell or multi-cell cultures were unable to preserve stemness state although various growth factors were introduced (stem cell factor, thrombopoietin, and FLT3L). The experimental results showed that decreasing in $CD34^+$ ratio is more likely to happen in lower cell density. The $CD34^+$ cell population was preserved by co-culturing with microencapsulated $CD34^+$ cells. Moreover, cell expansion increased significantly in the presence of microencapsulated $CD34^+$ cells (Figure S2). The number of loaded cells had a significant impact on the effectiveness of microencapsulated $CD34^+$ cells.

The paracrine release from microencapsulated cells was hypothesized to play an important role in promoting the proliferation and maintenance of cultured $CD34^+$ cells.

Additional experiments were conducted to address this hypothesis. The cells (Donor 6; lower well) were co-cultured with equivalent numbers of bare or encapsulated cells from an unrelated donor (Donor 5; upper well). On day 8, the cell viabilities of bare cells (lower well) were 94.4 ± 3.2; 91.7 ± 4.8 and 100 ± 0.0% for monoculture, co-culture with bare cells, and co-culture with microencapsulated cells. It was noticed that co-cultures with encapsulated or bare cells from the unrelated donor could improve the cell expansion (Figure S3A). Moreover, co-cultures produced higher percentages of $CD34^+$ cells than of monoculture (Figure S3B). These results suggested that co-culture with encapsulated cells, isolated from an unrelated donor, have beneficial effects for $CD34^+$ cell expansion and progenitor maintenance.

In the present study, thrombopoietin, which can promote cell proliferation and megakaryocytic differentiation [27], was added because platelet lysate alone could not provide enough growth factors for $CD34^+$ cell survival and proliferation in vitro (Figure S4). CD41 is expressed throughout megakaryocytic lineage and thus considered as a representative marker for megakaryotic differentiation [3,28]. It was found that bare and encapsulated cells have opposite effects on CD41 expression of co-cultured cells (Figure S5). Co-culture with bare cells from different donors suppressed CD41 expression of cultured cells (lower well). On the contrary, when the equivalent cells were encapsulated with alginate-chitosan, it could promote CD41 expression of cultured cells (lower well). To our knowledge, this is the first time such a paracrine effect is reported.

There are two possible mechanisms: (1) direct effect in which chitosan and/or alginate interact with thrombopoietin and subsequently promote megakaryocytic differentiation of external cells or (2) indirect effect in which chitosan and/or alginate interact with thrombopoietin or encapsulated cells and subsequently cause the entrapped cells to release paracrine factors that promote megakaryocytic differentiation of external cells. Further studies are needed to understand the detailed mechanism.

Encapsulation for transplantation was initially developed to improve allogeneic pancreas islet transplantation for diabetic patients [29]. Semi-permeable membrane provides mechanical protection and immune rejection for transplanted islets while nutrients and cellular metabolites freely diffuse through the membrane [30]. Several clinical trials have been performed, and the follow-up evaluation showed a safe and promising result of alginate-based microencapsulated islets for diabetic patients [30]. Inspired by these findings, we developed $CD34^+$ cells-loaded microcapsule to demonstrate paracrine factor delivery. The microcapsule possessed no toxicity for external cells. The 3D capsules could restrict cell growth of mesenchymal stem cells (MSCs) [31], myoblast and fibroblast cells [32], which was postulated due to limited nutrients/oxygen exchange. Limited cell growth is beneficial to prevent overpopulation inside the capsule as long as the cells are still capable of releasing paracrine factors. In addition, microencapsulation can be an advantage in facilitating scaled-up co-culture systems for HSC or progenitor expansion with bioreactors (Figure S6).

HSC homeostasis maintenance required paracrine factors secreted by niche cells to supply or feed the right signal for HSCs [33]. A study reported encapsulation of several cell types, including human amniotic-MSCs, umbilical cord-MSCs, and fibroblasts, to maintain $CD34^+$ cell expansion in vitro [31]. Their finding indicated that encapsulated MSCs or fibroblasts do not benefit $CD34^+$ cells proliferation [31]. Interestingly, improved cell proliferation of $CD34^+$ cells by co-culturing with osteosarcoma cell line has been reported [34]. Instead of employing other cells, our study used $CD34^+$ cells collected from unrelated donors as the source of paracrine factors required for hematopoietic progenitor maintenance. This system can be utilized to deliver paracrine factors for ex vivo HSC or progenitor expansion with bioreactor and further developed to support initial engraftment of allogeneic HSC transplantation.

4. Conclusions

A non-toxic double-layered microcapsule has been successfully fabricated with alginate-chitosan to encapsulate human $CD34^+$ progenitor cells. The microcapsules did not pose any

toxicity to external cells. Cell proliferation and progenitor maintenance were significantly improved when the cells from unrelated donors were cultured with $CD34^+$ cell-loaded microcapsules. The thrombopoietin-induced megakaryocytic differentiation was amplified when the cells were co-cultured with cell-loaded microcapsules, while it was repressed when co-cultured with non-encapsulated cells. Our study is the first proof-of-concept that microencapsulation can be utilized to deliver paracrine factors for ex vivo expansion of HLA-unmatched $CD34^+$ progenitors.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11177887/s1, Figure S1: A. Illustration of co-culture system of microencapsulated $CD34^+$ cells (6×10^3 cells/well) with bare $CD34^+$ cells from unrelated donors. B. Viable cell concentration of bare cells after co-culturing for 8 days, Figure S2: Fold expansions of $CD34^+$ cells (lower well) on day 8 post-culturing with and without cell-loaded microcapsules (3×10^4 cells/well), Figure S3: A. Viable cell concentration of bare cells (lower well) on day 8. B. Ratio of $CD34^+$ cell population on day 8. Bare cells and microencapsulated cells (Donor 5, upper well) were 3.0×10^4 cells/well. Initial seeding of bare cells (Donor 6; lower well) was 2.5×10^4 cells/ml. Donor 5 and 6 were unrelated patients. Figure S4: Effect of thrombopoietin (TPO) for $CD34^+$ cells cultured on platelet-lysate-(PL)-supplemented media, Figure S5: The $CD41^+$ cell population of bare cells (lower well) after 8-day monoculture or co-cultures in thrombopoietin-supplemented media; Illustration of co-culture systems with microcapsule-bioreactor and membrane insert.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analysed during this study are included in this published article and its supplementary information files.

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