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Original Article

Optimization of agarose–alginate hydrogel bead components for encapsulation and transportation of stem cells

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الملخص

أهداف البحث: تهدف هذه الدراسة إلى تحسين تكوين حبيبات هلامة "ألجينات الأغاروز" وعوامل التذويب الخاصة بها لاستخدامها المحتمل في نقل الخلايا الجذعية.

طرق البحث: تم اختبار تركيزات مختلفة من الأغاروز والألجينات وكلورايد الكالسيوم لبناء حبيبات هلامة مائية. تم تسجيل معدل التحلل ونسبة الانتفاخ لكل عينة هلامة مائية. تم استخدام هلامة "ألجينات الأغاروز" المحسنة لتغليف الخلايا الجذعية من أسنان بشرية لبنية متساقطة (شيد). تم أيضًا إجراء تحسين عوامل الإذابة واختبارها باستخدام الخلايا المغلفة بالهلامة المائية.

النتائج: تم إثبات أن التركيز المختار لمكونات هلامة "ألجينات الأغاروز" نجح في تغليف "شيد" والتي تظل قابلة للحياة حتى اليوم العاشر. تم تسجيل متوسط دقيقتين لتحلل الهلامة المائية التي تغلف "شيد"، باستخدام عامل إذابة يتكون من 100 ملي سيترات الصوديوم و 100 ملي مولار من إيديتات الكالسيوم ثنائي الصوديوم (إيديتا). تم تسجيل صلاحية خلية "شيد" التي تم إطلاقها بعد اليوم العاشر من التغليف بنسبة 29.1%.

الاستنتاجات: أوضحت الدراسة الحالية أن تركيزات كل من الأغاروز والألجينات وكلورايد الكالسيوم ترتبط بمعدل تحلل حبيبات الهلامة المائية ونسبة الانتفاخ. يمكن لـ "شيد" البقاء على قيد الحياة في بيئة هلامة مائية لمدة 10 أيام على الأقل دون تغيير وسط الاستزراع. يسمح تحسين عامل الذوبان بالتفكك المناسب لهيكل الهلامة المائية، مما يؤدي إلى إطلاق الخلايا الجذعية من حبيبات هلامة "ألجينات الأغاروز" بعد فترة النقل مع السماح للخلايا بالبقاء قابلة للحياة.

الكلمات المفتاحية: ألجينات؛ أغاروز؛ هلامة مائية؛ معدل التدهور؛ سترات الصوديوم؛ الخلايا الجذعية

Abstract

Hydrogels have potential uses in various biological applications because of their unique characteristics. Fine-tuning of agarose–alginate (Ag–Al) hydrogel components improves the mechanical characteristics of the final construct for cell encapsulation and transportation. Formulation of suitable dissolving agents may enable the release of encapsulated cells for further applications in laboratory or clinical settings.

Objectives: This study aimed at optimizing the composition of Ag–Al hydrogel beads and their dissolving agents for potential use in the transportation of stem cells.

Methods: Various agarose, alginate, and $CaCl_2$ concentrations were tested to construct hydrogel beads. The degradation rate and swelling ratio of each hydrogel sample were recorded. The optimized Ag–Al hydrogels were used for encapsulation of stem cells from human exfoliated deciduous teeth (SHED). Optimization of dissolving agents was performed and tested with the hydrogel-encapsulated cells. Data were statistically analyzed in SPSS.

Results: The selected concentration of Ag–Al hydrogels components was successfully demonstrated to encapsulate SHED, which remained viable until day 10. An average of 2 min was required for degradation of the hydrogel with encapsulated SHED by a dissolving agent consisting of 100 mM sodium citrate and 100 mM EDTA. The cell viability of SHED released after day 10 of encapsulation was 29.1%.

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Conclusion: Alteration of Ag-Al components has considerable influence on the mechanical properties of the constructed hydrogel. The feasibility of performing the optimized cell encapsulation protocol, as well as the dissolving step, may provide a useful guide for the transportation of viable cells between countries, for medical research.

Introduction

Hydrogels are highly hydrated materials composed of hydrophilic polymers that are produced from one or more monomers, which are cross-linked to form three-dimensional networks. The reaction produces a final product that can retain a substantial amount of water within the polymeric structure without dissolving in water and that mimics the structure of the extracellular matrix. These unique features have prompted research and development exploring the use of hydrogels for biomedical applications such as drug delivery, tissue regeneration, and cell encapsulation.[1−4] The ability of the components of a hydrogel to mimic the native environments of specific cell groups *in vitro* has enabled researchers to study cellular activities and physiology in cell biology, particularly in stem cells and cancerous cells. Hydrogels have exciting potential cell encapsulation applications, such as cell delivery in regenerative therapy or long-term transportation of cells between distant locations.

Currently, cryopreservation is widely used for long-term storage and transport of cells, however, it adds substantial cost to the delivery of stem cells.[5] Cryopreservation requires multiple steps and careful handling of the cells, including mixing cryoprotective agents with the cells, cooling the cells to a low temperature, warming the cells, and finally removing the cryoprotective agents from the cells after thawing.[6] Recent advances in hydrogel applications in biomedicine have indicated their potential for use in the transportation of stem cells without strict temperature control and transportation time limitations. The unique characteristics of hydrogels, such as high biocompatibility and elasticity, as well as their ability to resemble the extracellular matrix structure, may enable vast potential for application in regenerative therapy.[7] Hydrogels used for cell encapsulation can provide tissue-like water content, as well as easily tunable biochemical and mechanical properties that can accommodate the growth of various cell types, such as neural stem cells, human mesenchymal stem cells, and embryonic stem cells.[8]

Hydrogels synthesized from natural origins, such as alginate and agarose, have been postulated to offer more benefits than synthetic hydrogels. They are biocompatible and bioactive, and have similar mechanical properties to those of tissues.[9] Synthetic hydrogels may contain monomers or ligands that are crucial for the polymerization reaction of hydrogels. However, they may be toxic, particularly when the polymerization reaction is incomplete. Toxic moieties can leak from hydrogels and affect seeded cells, thus leading to cell toxicity and apoptosis.[10] Hence, synthetic hydrogels have limited applications *in vivo* because they may induce host responses and foreign body reactions. Ideally, hydrogels used for vehicles should also be highly biocompatible and possess minimal cytotoxicity after the encapsulation and release of stem cells.

Alginate has been extensively used in the preparation of hydrogel synthetic extracellular matrix. It is a naturally derived polysaccharide comprising β-D-mannuronic (M) and α-L-guluronic (G) residues in varying proportions. Gelation of alginate occurs in the presence of cations, such as calcium ions, and is reversible with the removal of calcium ions by mixing the gel with sodium chloride or sodium citrate.[11] Another commonly used component of hydrogels is agarose, a polysaccharide derived from marine red algae, consisting of alternating D-galactose and 3,6-anhydrous-L-galactose units. Agarose beads are highly porous, mechanically resistant, chemically and physically inert, and highly hydrophilic.[12] The combination of unique features of both alginate and agarose is believed to produce hydrogels with better quality that are suitable for stem cell transportation.

After the transportation process, stem cells must be released from the structure of the hydrogel for further cell expansion and manipulation. Sodium citrate has been proposed to dissolve ionically cross-linked hydrogels by exchanging calcium ions with monovalent cations and sodium ions. Furthermore, ethylenediaminetetraacetic acid (EDTA), a chelating agent that binds mostly bivalent ions (calcium), has been introduced to dissolve hydrogels. The correct formulation and optimization of the dissolving agent enables maximal control of the structural integrity and viability of encapsulated cells within the hydrogels, which serve as the delivery vehicle.

Therefore, this study was performed to construct hydrogel beads with optimum concentrations of alginate and agarose, that can support and accommodate the survival of stem cells without the need for regular culture medium changes and storage in low temperatures. Further optimization of the dissolving agent was performed with a combination of sodium citrate and EDTA to release the stem cells after the encapsulation process. The data gathered from this study may aid in better formulation of Ag−Al hydrogel components. The data may also support the potential use of Ag−Al hydrogel beads in the transportation of stem cells from remote locations, or between locations thousand miles apart.

Materials and Methods

Synthesis of Ag−Al hydrogel beads

The following steps were performed to fabricate the Ag−Al hydrogels. Sodium alginate (Sigma−Aldrich, UK), was dissolved at 50 mg/mL in deionized water under constant stirring at room temperature to prepare 5% alginate stock solution, which was autoclaved before use. An agarose stock solution at 3% (w/v) high melting agarose was prepared by

dissolving 1.5 g of agarose (Bioline, UK) in 48.5 mL deionized water; the solution was heated on a hot plate and occasionally stirred until completely dissolved. Several concentrations of agarose solutions were further prepared by dilution of the stock solution with 1% phosphate-buffered saline (PBS, pH 7.4) without $CaCl_2$ and $MgCl_2$ (Gibco/Invitrogen Life Technologies, USA). The temperature of the agarose solution was kept above 45 °C before being mixed with the alginate solution, because agarose has a specific gelation temperature.

Each concentration of agarose and alginate solution was mixed and stirred to form an Ag—Al solution, and the temperature of the final solution was maintained at 37 °C.

The Ag—Al solution was immediately loaded into syringes with 21 G hypodermic needles attached. The end of each needle was adjusted until it was 30 mm above the surface of the calcium chloride ($CaCl_2$) solution (Sigma—Aldrich, UK). The Ag—Al solution was added dropwise into 0.1, 0.2, or 0.3 M $CaCl_2$ solution under gentle stirring at room temperature for 8 min. The beads produced were filtered out and rinsed with PBS three times before being placed in cell culture medium at room temperature, before the next experiment. The experimental groups with different combinations of Ag—Al components used to construct the hydrogel beads are listed in Table 1.

Optimization of Ag—Al hydrogel components

To transport cells with the Ag—Al hydrogels, the primary aim of the preliminary work was identifying the suitable concentration of each hydrogel bead component for encapsulation. The constructed hydrogel was also required to be able to be degraded and to release the cells after arriving at

the desired destination. Hence, all constructed Ag—Al was subjected to analyses of swelling ratio, degradation, cell encapsulation capacity, and dissolution rate under an optimized concentration of citrate/EDTA solution.

Swelling ratio

The hydrogels were characterized by the swelling ratio, which describes the ability of an Ag—Al hydrogel to absorb water. The swelling ratio was measured by incubation of test specimens in 300 μL of deionized water at 37 °C for specific times (10, 20, 30, 40, 50, 60, 90, 120, 180, or 360 min). The Ag—Al hydrogel beads were oven-dried overnight at 40 °C before the experiments. The dried hydrogel beads were weighed, and the weight was denoted W_a. Hydrogel samples were transferred to plates containing PBS or deionized water and allowed to return to a swollen state. Excess water was carefully removed with soft, lint-free paper, the gels were weighed, and the weight was denoted W_b. The swelling ratio was calculated with the following formula (Evmenenko et al., 1999).[13]

$$Degree\ of\ swelling\ (\%) = [(W_b - W_a)/W_a] \times 100\%$$

Degradation test

The rate of degradation refers to the time required for each of the hydrogels to completely degrade until the weight of the wet hydrogel could not be measured. The weight loss of the hydrogel starting from the initial day to the final experimental day was recorded to calculate the rate of degradation as follows:

Table 1: Summary of the different concentrations of agarose, alginate, and $CaCl_2$ tested in Ag—Al bead production.

Samples	Agarose concentration (w/v)%	Alginate concentration (w/v)%	$CaCl_2$ concentration (M)
$Ag_{0.5} + Al_{1.0}$ (0.1 M $CaCl_2$)	0.5	1.0	100
$Ag_{1.0} + Al_{1.0}$ (0.1 M $CaCl_2$)	1.0	1.0	100
$Ag_{1.5} + Al_{1.0}$ (0.1 M $CaCl_2$)	1.5	1.0	100
$Ag_{2.0} + Al_{1.0}$ (0.1 M $CaCl_2$)	2.0	1.0	100
$Ag_{0.5} + Al_{1.5}$ (0.1 M $CaCl_2$)	0.5	1.5	100
$Ag_{1.0} + Al_{1.5}$ (0.1 M $CaCl_2$)	1.0	1.5	100
$Ag_{1.5} + Al_{1.5}$ (0.1 M $CaCl_2$)	1.5	1.5	100
$Ag_{2.0} + Al_{1.5}$ (0.1 M $CaCl_2$)	2.0	1.5	100
$Ag_{0.5} + Al_{1.0}$ (0.2 M $CaCl_2$)	0.5	1.0	200
$Ag_{1.0} + Al_{1.0}$ (0.2 M $CaCl_2$)	1.0	1.0	200
$Ag_{1.5} + Al_{1.0}$ (0.2 M $CaCl_2$)	1.5	1.0	200
$Ag_{2.0} + Al_{1.0}$ (0.2 M $CaCl_2$)	2.0	1.0	200
$Ag_{0.5} + Al_{1.5}$ (0.2 M $CaCl_2$)	0.5	1.5	200
$Ag_{1.0} + Al_{1.5}$ (0.3 M $CaCl_2$)	1.0	1.5	200
$Ag_{1.5} + Al_{1.5}$ (0.2 M $CaCl_2$)	1.5	1.5	200
$Ag_{2.0} + Al_{1.5}$ (0.2 M $CaCl_2$)	2.0	1.5	200
$Ag_{0.5} + Al_{1.0}$ (0.3 M $CaCl_2$)	0.5	1.0	300
$Ag_{1.0} + Al_{1.0}$ (0.3 M $CaCl_2$)	1.0	1.0	300
$Ag_{1.5} + Al_{1.0}$ (0.3 M $CaCl_2$)	1.5	1.0	300
$Ag_{2.0} + Al_{1.0}$ (0.3 M $CaCl_2$)	2.0	1.0	300
$Ag_{0.5} + Al_{1.5}$ (0.3 M $CaCl_2$)	0.5	1.5	300
$Ag_{1.0} + Al_{1.5}$ (0.3 M $CaCl_2$)	1.0	1.5	300
$Ag_{1.5} + Al_{1.5}$ (0.3 M $CaCl_2$)	1.5	1.5	300
$Ag_{2.0} + Al_{1.5}$ (0.3 M $CaCl_2$)	2.0	1.5	300

$$\text{Rate of degradation} = \frac{Ax - Ao \text{ (weight)}}{x - o \text{ (number of days)}}$$

where Ao is the average weight of the hydrogel bead at day 0, and Ax is the average weight of the hydrogel bead at day X.[14]

Analysis of hydrogel surface with scanning electron microscopy (SEM)

$Ag_{2.0}/Al_{1.5}$ (0.2 M $CaCl_2$) and $Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$) in the hydrogel beads' surface structure was observed with a Quanta FEG 450 instrument in the SEM Laboratory, School of Health Sciences, USM. The beads were kept in their natural state without freeze-drying. Both samples were examined at a voltage of 5.0 kV under low vacuum. The beads were coated with gold at a thickness of 22.3 nm.

Cell culture

Stem cells from human exfoliated deciduous teeth (SHED) purchased from AllCells USA were revived from the cryovial and maintained in culture before encapsulation. SHED from passage 14 were used for encapsulation into Ag—Al hydrogel beads. Cells were culture expanded in alpha-modified Eagle's medium (αMEM) (Life Technologies, USA) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, USA) and 1% penicillin—streptomycin. SHED were further incubated at 37 °C in a 5% humidified incubator, and the medium was changed every 2 or 3 days. At 70% confluency, the cells were trypsinized and further passaged or used for experiments.

Cell count

Each cell pellet was transferred into 1 mL of culture medium and mixed thoroughly; 10 μL of cells was then added into 10 μL of trypan blue solution and gently mixed. A final 10 μL solution from the mixture was transferred into the hemocytometer counter chamber. The numbers of viable cells and total cells were counted, and the percentage of viable cells was then calculated as follows.[15]

$$\% \text{ Cell viability} = \frac{\text{Number of viable cells}}{\text{Number of total cells}} \times 100.$$

Encapsulation of stem cells

A total of 1 mL of $Ag_{0.1}/Ag_{1.5}$, 0.3 M $CaCl_2$ hydrogel mixture with SHED was prepared. The concentration was chosen to encapsulate the SHED after confirmation of a successful dissolution test with sodium citrate—EDTA. Briefly, 450 μL of cell suspension containing 5×10^5 cells was mixed with 200 μL of 1.5% sodium alginate solution in a 1.5 mL microcentrifuge tube at room temperature. The cell suspension was added to 350 μL of 0.1% agarose solution at 35—40 °C to avoid cell damage. The cell-agarose-alginate gel solution was then loaded into a 10 mL syringe with a 21 G hypodermic needle attached, which was fixed to a tripod stand. The end of the needle was kept 30 mm above the surface of the $CaCl_2$ solution. The cell-gel solution was added dropwise into the $CaCl_2$ solution under gentle stirring at room temperature for 8 min to produce SHED encapsulated Ag—Al hydrogels. The beads were washed three times with PBS, then placed in four culture dishes containing α-MEM culture medium and were left on a laboratory bench at room temperature without changing the culture medium for 10 days, simulating the approximate transportation time (for cell delivery across different continents in the world).

Optimization of Ag—Al hydrogel dissolving solution

Sodium citrate (10.0, 50.0, 100.0 and 150.0 mM) and EDTA (10, 50 and 100 mM) were prepared by mixture of sodium citrate dihydrate 0.5% (v/v) and EDTA 0.2% (v/v) (Biochrom, USA) with distilled water. The final dissolving solutions were prepared by mixture of different combinations of citrate and EDTA at different concentrations with 0.15 M NaCl, pH 8.2.

Dissolution test with sodium citrate—EDTA

The dissolution rate of Ag—Al hydrogels was defined as the decrease in weight during exposure to citrate/EDTA. The Ag—Al hydrogels were weighed and placed in a beaker filled with 30 mL of degradation agent and gently shaken. The time taken for the hydrogel beads to fully disintegrate was recorded. After the SHED encapsulation protocol, the Ag—Al hydrogel beads containing entrapped stem cells were kept at room temperature. At days 1, 3, 7, and 10 post-encapsulation, the beads were treated with a dissolving agent containing $\text{citrate}_{10 \text{ mM}}/\text{EDTA}_{10 \text{ mM}}$, or $\text{citrate}_{100 \text{ mM}}/\text{EDTA}_{100 \text{ mM}}$, respectively.

Cell viability testing after release from Ag—Al hydrogels

SHED encapsulated Ag—Al hydrogels were kept at room temperature without changing of the culture medium. After 10 days, all Ag—Al beads were collected and dissolved with $\text{citrate}_{100 \text{ mM}}/\text{EDTA}_{100 \text{ mM}}$ to determine the percentage cell viability via trypan blue exclusion assays. SHED were released from the hydrogel constructs by centrifugation at 8000 rpm for 5 min. A cell count was performed, and the number of viable cells was recorded.

Data analysis

The data were analyzed with one-way ANOVA in SPSS (23.0, SPSS Inc., Chicago, IL, USA). All experiments were performed three times with triplicates (n = 3), and the data are expressed as mean ± SEM, with a significance threshold set at $p < 0.05$.

Results

Synthesis and general appearance of Ag—Al hydrogel beads

The Ag—Al hydrogel combinations proposed in this study demonstrated the successful formation of hydrogel beads, although the method produced irregular, inconsistent spherical bead structures, as shown in Figure 1(a—b). The
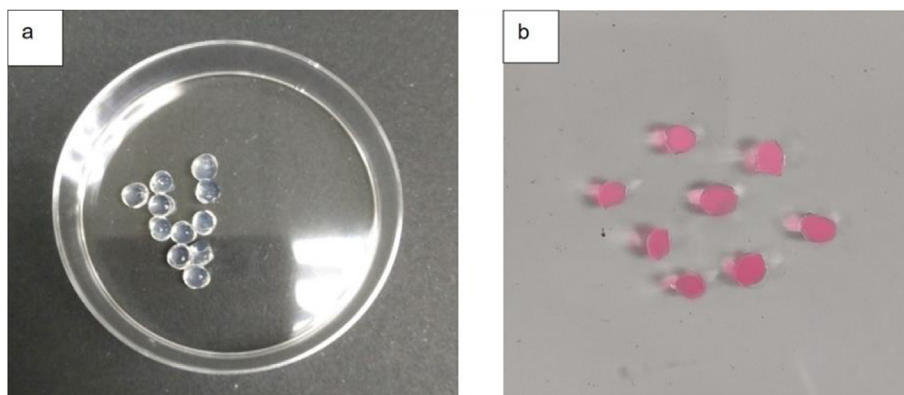
Figure 1: Representative image of the gross appearance of formed hydrogel beads (in the swollen state). (a) Unstained Ag−Al beads, (b) Ag−Al hydrogel beads constructed with the addition of αMEM culture medium.

average size of a single Ag−Al hydrogel bead synthesized was recorded at 2.2 mm × 3.4 mm (length × width), with an average wet weight of 0.69 mg.

Swelling ratio of Ag−Al hydrogel beads

The swelling ratio of Ag−Al hydrogel beads constructed with different concentrations of $CaCl_2$ is shown in Figure 2. The solid lines indicate a 1% alginate concentration, whereas the dotted lines indicate a 1.5% alginate concentration. Two-independent ANOVAs indicated no significant differences in the degradation rate and swelling ratio of different Ag−Al hydrogel concentrations. The plotted data also demonstrated that a higher concentration of $CaCl_2$ may be closely associated with a lower swelling ratio. Overall, hydrogel beads made of 1.5% alginate solution exhibited a lower swelling ratio than hydrogel beads made of 1% alginate, regardless of the agarose and $CaCl_2$ used.

Degradation rate of Ag−Al hydrogel beads

Each bar represents samples with different concentrations of alginate, agarose and calcium chloride used to produce the hydrogel beads. The data indicated consistent degradation of material over the experimental period. The physiologic degradation rate of the constructed Ag−Al hydrogel beads is shown in Figure 3. Variations in the degradation rate were recorded for each component used to construct the Ag−Al hydrogel beads. The most striking finding was observed with a combination of $Ag_{1.0}/Al_{1.5}$ (0.1 M $CaCl_2$), which showed the highest rate of degradation, 0.218 mg/day. With a 0.1 M $CaCl_2$ concentration, 1.5% alginate showed a relatively higher degradation rate than 1.0% alginate. However, a similar finding was not observed with samples constructed with 0.2 M and 0.3 M $CaCl_2$. In general, changes in agarose concentration may thus have a profound effect on the degradation rate, as observed in all groups of tested $CaCl_2$ concentrations.

Encapsulation of SHED in Ag−Al hydrogel beads

Observations of SHED morphology demonstrated that the cultured cells exhibited regular mesenchymal stem cell,

spindle-like shape features, as shown in Figure 4a. After swelling ratio and degradation rate assessment, only three combinations of Ag−Al concentrations were selected for the initial cell encapsulation process. The $CaCl_2$ concentrations were as follows: $Ag_{1.0}/Al_{1.5}$ (0.1 M $CaCl_2$), $Ag_{2.0}/Al_{1.5}$ (0.2 M $CaCl_2$), and $Ag_{1.5}/Al_{1.0}$ (0.3 M $CaCl_2$). The selection of Ag−Al combinations was made on the basis of the recorded degradation rate and swelling ratio. The selected combination of Ag−Al hydrogels—$Ag_{1.0}/Al_{1.5}$ (0.1 M $CaCl_2$), $Ag_{2.0}/Al_{1.5}$ (0.2 M $CaCl_2$), and $Ag_{1.5}/Al_{1.0}$ (0.3 M $CaCl_2$)—was demonstrated to successfully encapsulate SHED. Figure 4b and c displays representative images of encapsulated SHED in $Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$) hydrogel beads without any staining material under different magnifications. However, our observations indicated that the Ag−Al hydrogels with a high swelling ratio tended to burst too quickly during the storage or incubation period, thus making them relatively less suitable as a transportation medium.

Dissolution rate of Ag−Al hydrogel beads with sodium citrate and EDTA

In an ideal Ag−Al hydrogel formulation for stem cell transportation, the material used should easily disintegrate after reaching the destination. Hence, all three selected Ag−Al concentrations were further tested with different citrate/EDTA concentrations to ensure the dissolution of the structure after the SHED encapsulation process. By 10 days after cell encapsulation, the selected Ag−Al hydrogels were all degraded with the citrate/EDTA solution. However, none of the Ag−Al combinations were considered acceptable, because the formed beads remained intact in the dissolving solution, even after 2 h of constant stirring. The data gathered from the degradation protocol with citrate/EDTA demonstrated the difficulty of dissolving the Ag−Al hydrogels with a higher percentage of agarose. Hence, a new combination of Ag−Al hydrogel with low agarose content was tested. The new Ag−Al combination, $Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$), is represented by a red line in Figures 1 and 2, and showed a swelling ratio of 19.5 and a degradation rate of 0.486 mg/day. $Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$) was added to the existing list of Ag−Al/$CaCl_2$ combinations after a successful dissolution rate test with citrate/EDTA.
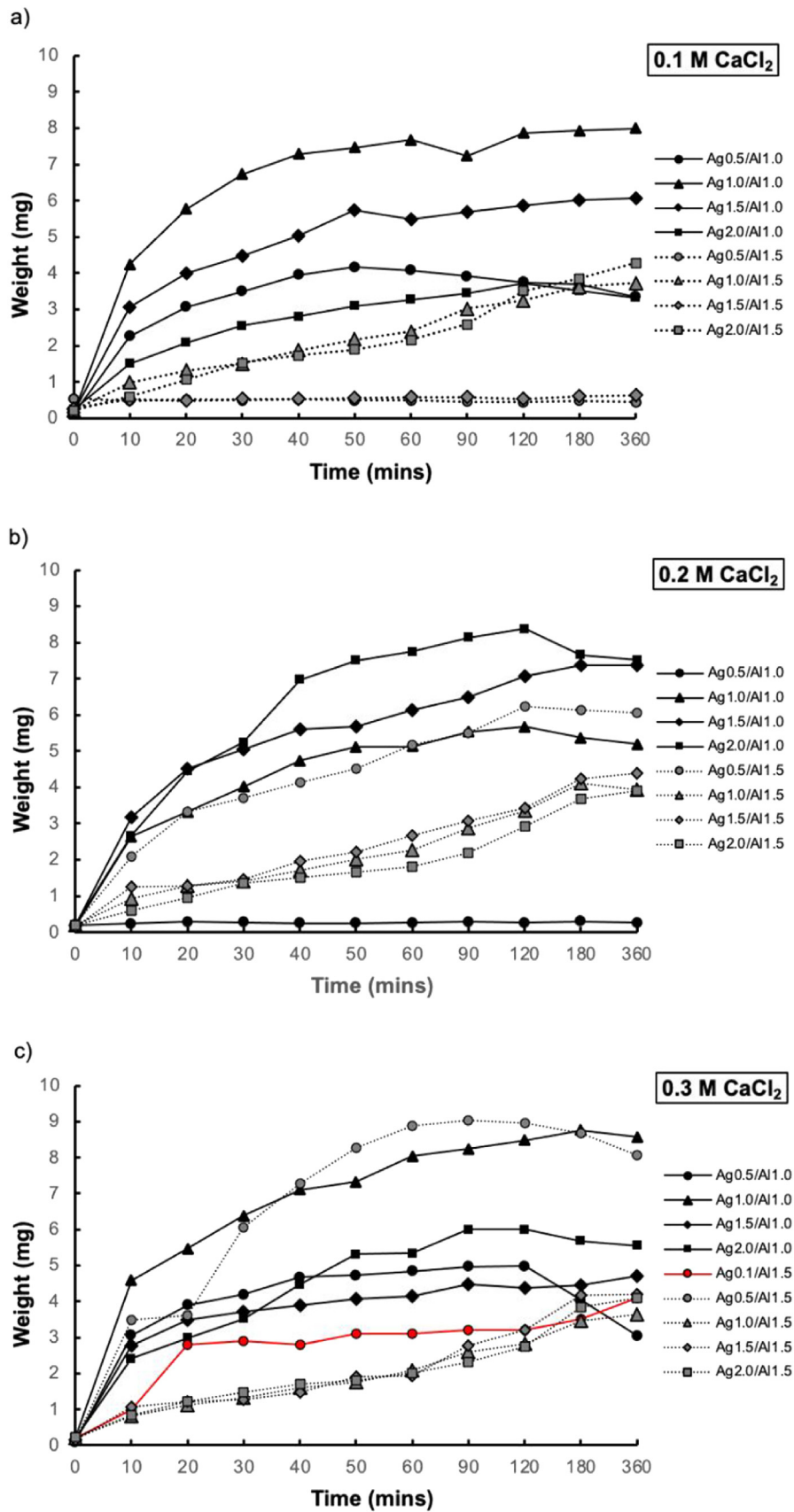
Figure 2: Swelling ratios of Ag−Al hydrogel beads. The Ag−Al beads were constructed with (a) 0.1 M, (b) 0.2 M, and (c) 0.3 M CaCl$_2$. The solid and dotted lines represent 1.0% and 1.5% alginate concentrations, respectively. The red line represents the optimized samples, Ag$_{0.1}$/Al$_{1.0}$ (0.3 M CaCl$_2$).
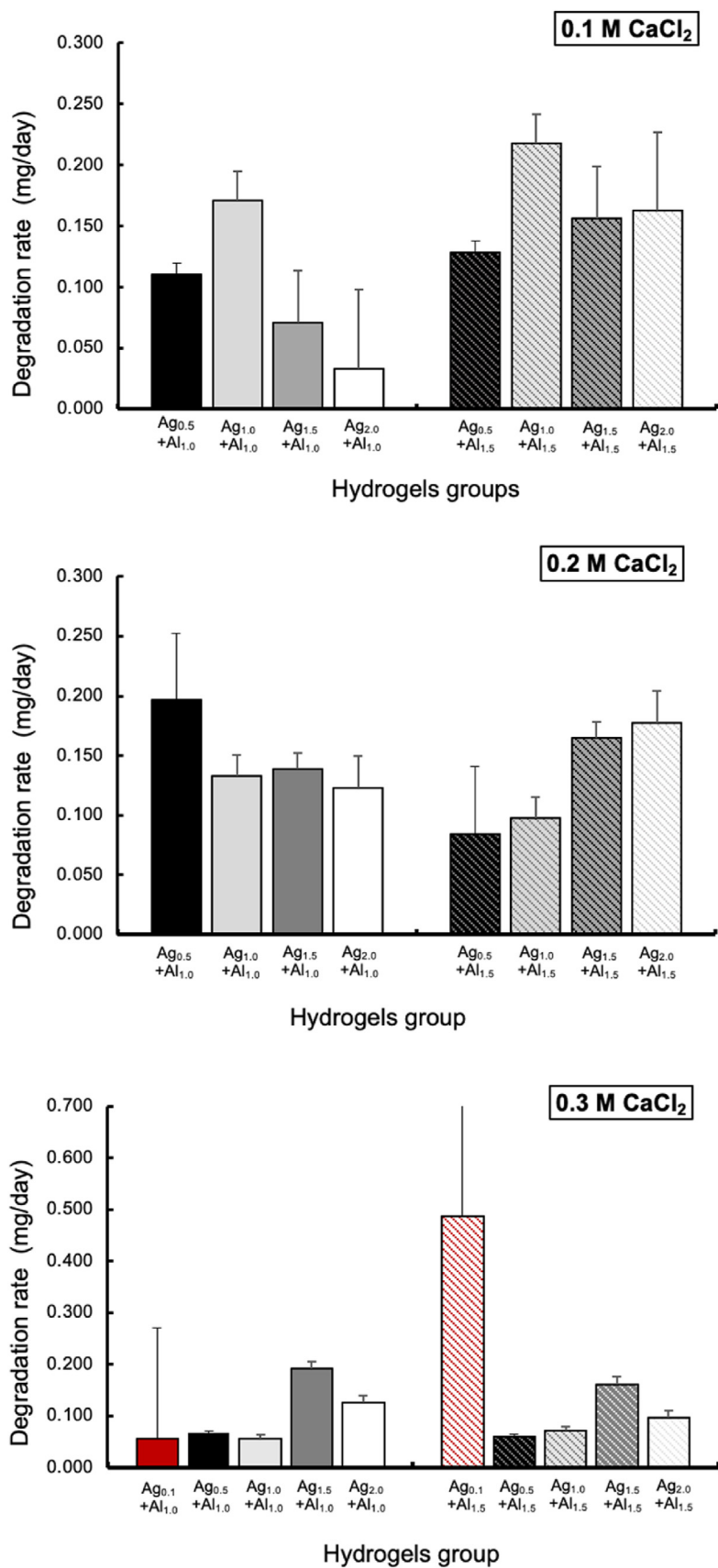
Figure 3: Bar chart representing the degradation rate (mg/day) of Ag−Al hydrogel beads constructed with 0.1 M, 0.2 M or 0.3 M $CaCl_2$, and monitored for a minimum of 10 days (n = 10). Data are presented as mean + SEM. The red bars (solid and lined) represent the optimized samples, $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$).
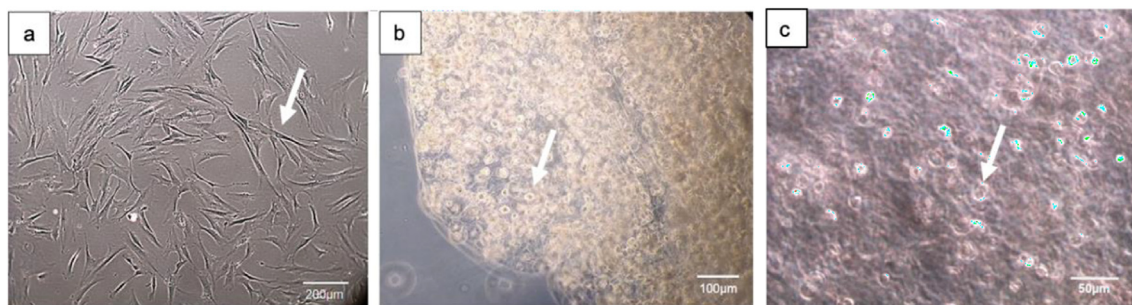
Figure 4: Morphological observation of SHED taken from passage 14. (a) Image captured at 60% confluency, in which SHED show a spindle-shaped morphology. (b) Representative image of SHED encapsulated in $Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$) hydrogel without staining, 24 h after the encapsulation process. (c) Representative image of SHED encapsulated in $Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$) hydrogel, without staining, at higher magnification. Arrows point toward the cells.

Table 2: Degradation times of alginate hydrogels and $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) beads with citrate and EDTA.

Dissolving solution	Degradation time (min), n = 3	
	Pure alginate	$Ag_{0.1}/Al_{1.5}$ (0.3 M $CaCl_2$)
$Citrate_{10\ mM}/EDTA_{10\ mM}$	13.2	[a]12.1
$Citrate_{10\ mM}/EDTA_{50\ mM}$	11.4	10.3
$Citrate_{10\ mM}/EDTA_{100\ mM}$	9.5	7.5
$Citrate_{50\ mM}/EDTA_{10\ mM}$	13.0	10.0
$Citrate_{50\ mM}/EDTA_{50\ mM}$	12.1	7.2
$Citrate_{50\ mM}/EDTA_{100\ mM}$	7.5	6.2
$Citrate_{100\ mM}/EDTA_{10\ mM}$	[a]13.8	8.5
$Citrate_{100\ mM}/EDTA_{50\ mM}$	11.4	6.2
$Citrate_{100\ mM}/EDTA_{100\ mM}$	12.4	[b]4.0
$Citrate_{150\ mM}/EDTA_{10\ mM}$	11.0	7.4
$Citrate_{150\ mM}/EDTA_{50\ mM}$	8.2	6.7
$Citrate_{150\ mM}/EDTA_{100\ mM}$	[b]6.0	5.2

[a]Longest and [b]shortest taken to dissolve the Ag−Al hydrogel.

Assessment of citrate/EDTA combination as the dissolving solution

The dissolution rate of pure alginate and $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) hydrogel beads was tested with different combinations of citrate/EDTA concentrations, as shown in Table 2. All tested combinations of citrate/EDTA successfully disintegrated the pure alginate hydrogels within an average of 13 min with $EDTA_{10\ mM}$, regardless of the citrate concentration applied. The shortest time
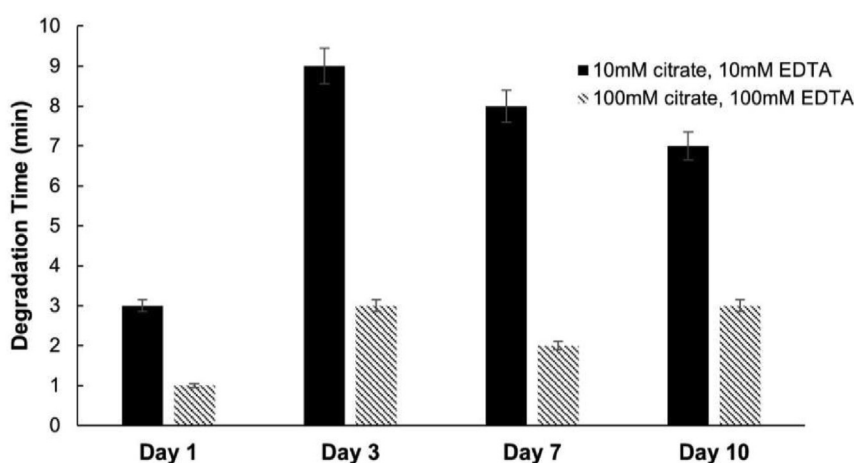

Figure 5: Degradation times of $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) hydrogels with sodium citrate/EDTA after encapsulation of SHED. The degradation times of Ag−Al hydrogels with citrate and EDTA were recorded at days 1, 3, 7 and 10 post-cell encapsulation. $Citrate_{100\ mM}/EDTA_{100\ mM}$ concentrations exhibit excellent properties as a dissolving solution to disintegrate $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$).
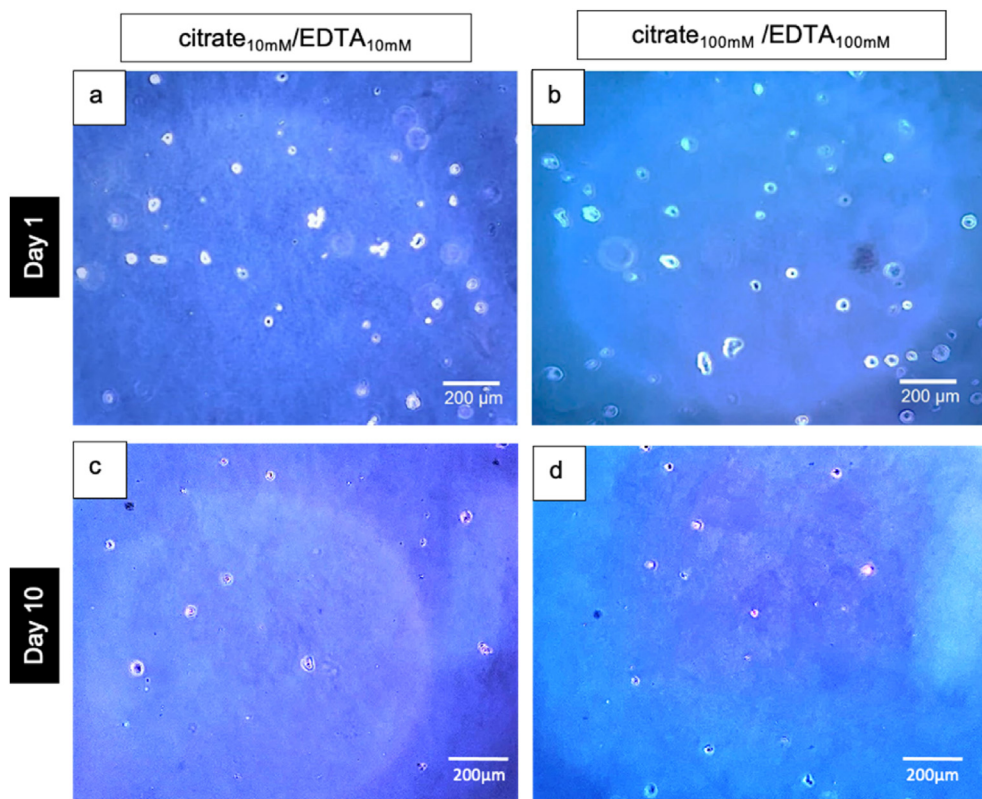
Figure 6: Assessment of cell viability after the encapsulation process. Representative image of SHED released from the $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) hydrogels after the dissolution step (a) at day 1 post-encapsulation in $citrate_{10\ mM}/EDTA_{10\ mM}$, (b) at day 1 post-encapsulation in $citrate_{100\ mM}/EDTA_{100\ mM}$, viewed with light microscopy, (c) at day 10 post-encapsulation, dissolved in $citrate_{10\ mM}/EDTA_{10\ mM}$, and (d) at day 10 post-encapsulation, dissolved in $citrate_{100\ mM}/EDTA_{100\ mM}$ (10× magnification).

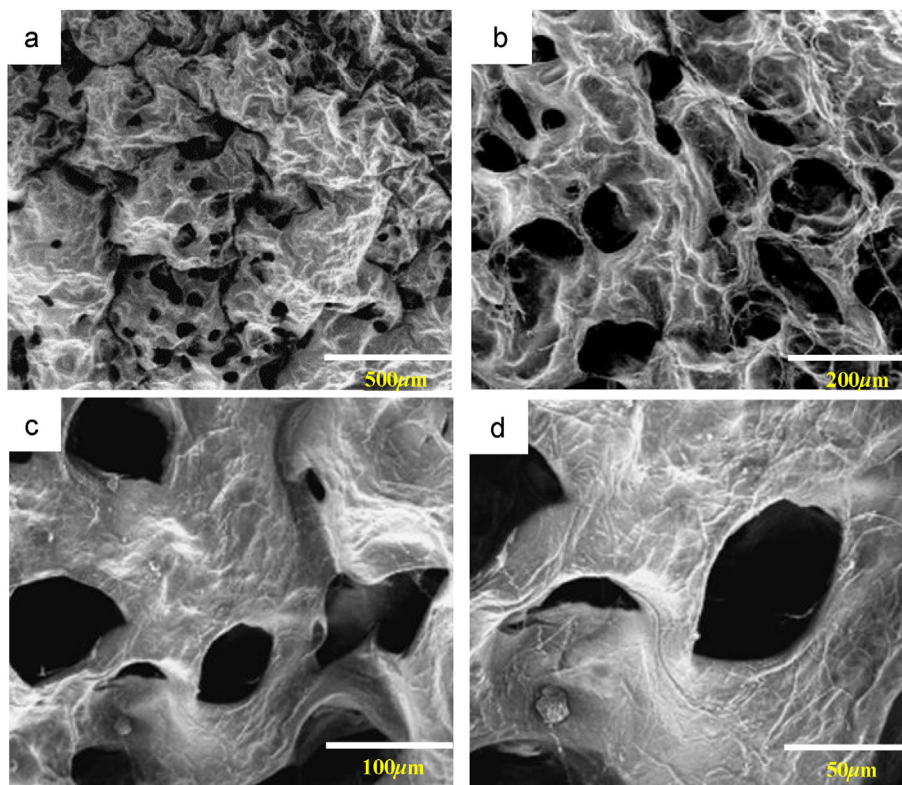**Figure 7:** SEM images of $Ag_{2.0}/Al_{1.5}$ (0.2 M $CaCl_2$) hydrogel beads at different magnifications.

observed was 6 min with citrate$_{150 \text{ mM}}$/EDTA$_{100 \text{ mM}}$. In contrast, a combination of citrate$_{10 \text{ mM}}$/EDTA$_{10 \text{ mM}}$ required 12 min to dissolve the Ag$_{0.1}$/Al$_{1.0}$ (0.3 M CaCl$_2$) hydrogel beads, whereas the shortest degradation time was recorded at 4 min with citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$.

Degradation of Ag$_{0.1}$/Al$_{1.0}$ (0.3 M CaCl$_2$) hydrogel after SHED encapsulation

The degradation times of selected hydrogel beads Ag$_{0.1}$/Al$_{1.0}$ (0.3 M CaCl$_2$) after encapsulation of SHED are shown in Figure 5. The solid bar represents citrate$_{10 \text{ mM}}$/EDTA$_{10 \text{ mM}}$, whereas the patterned bar represents citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$ as dissolving agents. The degradation times for encapsulated SHED within Ag−Al hydrogel beads were two times longer in citrate$_{10 \text{ mM}}$/EDTA$_{10 \text{ mM}}$ than citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$.

Cell viability after release from the structure of the hydrogel

The cell viability was assessed on day 10 after SHED encapsulation in Ag$_{0.1}$/Al$_{1.5}$ (0.3 M CaCl$_2$) hydrogel beads, which were kept at room temperature throughout the experimental period. On day 10, the Ag−Al hydrogels were dissolved in either citrate$_{10 \text{ mM}}$/EDTA$_{10 \text{ mM}}$ or citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$. However, for cell viability analysis, only citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$ was selected for cell release, owing to the shorter degradation time. Figure 6 shows representative images of viable SHED released from the hydrogel beads after the dissolution step with citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$, at day 1 post-encapsulation. The cell viability rate 10 days after encapsulation with release with citrate$_{100 \text{ mM}}$/EDTA$_{100 \text{ mM}}$ (n = 3) was 29.1%.

Surface morphology analysis by scanning electron microscopy

Two concentrations of Ag−Al hydrogel beads, Ag$_{2.0}$/Al$_{1.5}$ (0.2 M CaCl$_2$) and Ag$_{0.1}$/Al$_{1.5}$ (0.3 M CaCl$_2$), were subjected to SEM analysis of surface morphology. However, the SEM images of the Ag$_{0.1}$/Al$_{1.5}$ (0.3 M CaCl$_2$) hydrogel could not be obtained because low resistance to electron heat caused the samples to shrink too rapidly for images to be captured. However, SEM images of hydrogel beads constructed with Ag$_{2.0}$/Al$_{1.5}$ (0.2 M CaCl$_2$) components were successfully captured, as shown in Figure 7(a-d). Briefly, the composite hydrogel network of Ag$_{2.0}$/Al$_{1.5}$ (0.2 M CaCl$_2$) exhibited a highly compact and rough surface structure with a pore size of approximately 50 μm. The Ag$_{2.0}$/Al$_{1.5}$ (0.2 M CaCl$_2$) hydrogel also showed a homogeneous microstructure with inter-connectively and well-defined porosity, and a relatively higher number of pores.

Discussion

The data gathered in this study indicated that changes in polymer composition alter both the degradation rate and swelling ratio of Ag−Al hydrogel beads. This finding is in agreement with data obtained from another study suggesting that the manipulation of hydrogel composition can enhance the properties and performance of the resultant hydrogel in terms of physical and mechanical strength.[16] The change in weight for each composition of Ag−Al hydrogel beads over 10 days of the observation period was recorded and calculated as the degradation rate (mg/day). One comprehensive study on the mechanical properties of alginate hydrogel beads has demonstrated that the rapid gelation rate with CaCl$_2$ solution leads to variations in crosslinking densities and the polymer concentration gradient across the gel beads.[17] The formed alginate gel beads were lumpy and heterogeneous in structure, thus resulting in weak and inconsistent mechanical properties. This rapid gelation behavior of CaCl$_2$ is further complicated by the gradual reversal of ionic crosslinking of calcium alginate chains in the presence of sodium ions, thus leading to a substantial loss of mechanical strength.[18]

The optimized composition of hydrogels successfully produced a homogeneous and stable surface microstructure of the Ag−Al hydrogel. The cross-linking agent is a critical factor in the gelation kinetics and the resulting gel's structural characteristics.[19] In addition, a combination of alginate and agarose as a biocomposite hydrogel, as compared with pure agarose or alginate alone, markedly improves the mechanical properties.[20] Comparative findings from another study have also indicated that components of alginate and agarose in the hydrogel structure contribute to the formation of more refined 3D constructs with improved consistency in porosity and interconnectivity, as well as visible pores formation.[21] As the pore size increases, larger substances can diffuse through the hydrogels. The pores whose sizes are equivalent to or slightly larger than those of the cells, from 1 to 20 μm, may provide attachment support for cell growth, thus regulating cell morphology and providing microchannels for nutrient diffusion.[22] Larger pores of approximately 20−100 μm may also provide sufficient space for stem cell proliferation and tissue growth, as well as larger channels for nutrient diffusion.[23]

Alteration of the agarose concentration directly affected the degradation rate, in comparison to alteration of alginate and CaCl$_2$ alone, a finding contrasting with those from another study reporting that agarose gel is mechanically stable under normal conditions.[12] In this study, the agarose might have served as a filler component to stabilize the alginate polymer, thus improving the mechanical properties of the hydrogel. Moreover, alginate has been suggested to be the critical material during the encapsulation process, because it may act as a liquid precursor to suspend cells before encapsulation.[24]

However, this study indicated that high agarose content in Ag−Al hydrogels could not be degraded by a dissolving agent of sodium citrate and EDTA, possibly because agarose can be degraded only in the presence of agarase or when the temperature exceeds the melting point of >80−90 °C.[25] Hence, the melting point method is not practical, because the temperature is too high for the cells to remain viable, and the hydrogel structure will denature several degrees above 40 °C. Our findings also suggested that agarose concentration substantially affects hydrogel stability under increasing temperatures. For example, the SEM image of Ag$_{0.1}$/Al$_{1.0}$ (0.3 M CaCl$_2$) could not be captured because

low resistance to electron heat resulted in rapid shrinkage before the image could be captured. Another experimental study performing agarose analysis by SEM has reported that pore size increases with the setting temperature, owing to the melting of the weak junctions.[26] Similarly, we propose that low agarose content correlates with greater pore size in the $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) hydrogels and melting of the weak gel structure.

Therefore, we produced a new hydrogel bead formulation with the following combination of $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) of calcium chloride and tested it with the dissolving agent. These hydrogel beads were successfully dissolved by sodium citrate/EDTA solution within 4−12 min. The present finding was also consistent with data obtained from another study suggesting that the disintegration speed of alginate hydrogels increases with higher sodium citrate concentration.[11]

After the optimization protocol, SHED were successfully encapsulated in the Ag−Al hydrogel construct. However, a further comparison between the Ag−Al hydrogel alone and Ag−Al hydrogel with encapsulated SHED indicated a much lower degradation time for the latter, although the degradation test was performed with a similar concentration of the dissolving agent. Future studies are necessary to understand the mechanism underlying this observation.

Several factors may influence hydrogel degradation. A study has demonstrated that biodegradable polyethylene glycol hydrogels containing polylactic acid-degradable linkages degrade significantly faster when cultured in serum-containing cell culture medium than when placed in a phosphate-buffered saline solution.[26] Moreover, the presence of cells may slow the disintegration process of biodegradable polyethylene glycol gels,[27] a finding partly attributed to differences in water content between cell-laden and cell-free gels. Hence, many factors must be considered in analyzing the degradation rate, particularly the cell culture environment and the type of degradable linkages within the hydrogels.

Our observations made over a 10 day cell encapsulation period demonstrated that the hydrogel beads with a composition of $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) of calcium chloride dissolved up to 63%, a rate comparable to that in a study reporting that calcium chloride-crosslinked alginate scaffolds degrade up to 40% after 15 days.[28] In addition, changing the concentrations of alginate, agarose, and $CaCl_2$ alone did not directly affect the degradation rate, similarly to findings from another study proposing that agarose gel is relatively mechanically stable under normal conditions.[12]

The swelling ratio of the Ag−Al hydrogel beads was plotted against the weight of dried Ag−Al hydrogel beads after resuspension in water at different time intervals for a total of 360 min. The degree of swelling, indicating the stiffness and porosity of the gel matrix, was largely affected by the molecular network formed within the hydrogel matrix. From this study, the optimized concentration of hydrogel beads formed with $Ag_{0.1}/Al_{1.0}$ (0.3 M $CaCl_2$) demonstrated a swelling ratio of 19.5, a value lower than the average in a previous study. Again, according to our overall results, changes in agarose concentration did not considerably affect the stability of the hydrogel, because agarose does not show any swelling or shrinkage in water.[29] In contrast, higher alginate concentrations may decrease the swelling of

the hydrogel beads, in agreement with results from another study indicating that the network of the polymer is tighter with a high concentration of alginate.[30]

Cell viability assessment demonstrated that SHED can survive within Ag−Al hydrogels for 10 days without a requirement for nutrients and temperature control. The viability percentage of the released SHED was 29.1%, which is quite low with respect to the survival rates of 74% of mouse embryonic stem cells and 80% of human mesenchymal stem cells stored inside alginate hydrogels for 5 days under ambient conditions in a sealed cryovial.[31] The lower rate of cell viability observed in this study might have been due to the longer encapsulation time applied than the shorter experimental periods in prior studies.

The data from the present study suggested the potential for use of Ag−Al hydrogels in the transportation of stem cells. Similarly, another study has demonstrated that hydrogels allow cells to survive outside laboratory settings without the complex and bulky infrastructure required for cryopreservation.[32] Even so, proper testing of cell viability must be performed in the future, to provide detailed characterization of cells after the encapsulation and degradation process. Therefore, in future studies, proper cell viability tests, such as immunocytochemistry, antibody staining, and MTT assays, may be considered to investigate the detailed effects of encapsulation and degradation on cell behavior, proliferation, and differentiation, particularly after the long-term cell encapsulation period. Furthermore, a study of the structural and mechanical properties of the Ag−Al hydrogel beads may be an important area of exploration to provide in-depth knowledge of hydrogels' potential for research and clinical applications.

Conclusion

In summary, this study demonstrated that Ag−Al hydrogels can accommodate and support the survival of dental stem cells without requirements for nutrients and temperature control for at least 10 days. SHED encapsulated within the Ag−Al hydrogel matrix were successfully released by sodium citrate/EDTA and remained viable. Hence, the present data support the potential use of Ag−Al hydrogels in the transportation of stem cells for research and cell-based therapy.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

Ethical approval was granted for this study from the Human Research Ethics Committee, Universiti Sains

Malaysia on 5 September 2018 with an approval number: USM/JEPeM/18080397.

Authors contributions

MKXP conducted research, collected research material, and wrote the initial draft. HWZ conducted research, collected research material, and organized data. NSA performed and validated the cell culture protocol. NAH organized and wrote the final draft. NFG provided data from electron microscopy analysis. NY designed the study, approved the final draft, and proofread the manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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