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Multilayered Core-Shell Microcapsules with Liquid or Semi-Liquid Cores: A Breakthrough in Cell Therapy for Bone Regeneration

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ABSTRACT

Cell therapy has emerged as one of the most promising strategies for treating various defects and injuries, particularly in bone tissue. Encapsulation of mesenchymal stem cells within multilayered microcapsules addresses challenges related to limited cell sources and host immune responses. Furthermore, liquid-core microcapsules demonstrate superior diffusion and material exchange compared to solid or semi-liquid core counterparts due to their fluidic nature. In this study, 11-layered alginate (1%)–gelatin (2.5%) microcapsules incorporating nano-hydroxyapatite (nHA) (0.5%) with liquid and semi-liquid cores were fabricated using layer-by-layer deposition of alginate and chitosan, and their properties were evaluated. Semi-liquid core microcapsules exhibited 2.5-fold greater mechanical strength than liquid-core microcapsules, as assessed by rotational stress tests. Human mesenchymal stem cells encapsulated within these microcapsules were analyzed using MTT and ALP assays and alizarin red staining. Results revealed significantly enhanced cell proliferation and faster osteogenic differentiation in liquid-core microcapsules, with ALP activity peaking at 33 U/L on day 7 and maximum calcium deposition observed on day 21. These findings highlight the potential of liquid-core microcapsules for efficient bone regeneration therapies.

What is "already known":	• Encapsulation of mesenchymal stem cells within multilayered microcapsules addresses challenges related to limited cell sources and host immune responses. • Multilayered microcapsule (with liquid or semi-liquid core) is a novel method for enhancing cell therapy in bone regeneration, particularly by encapsulating mesenchymal stem cells. • Liquid-core microcapsules (LCM) demonstrate superior diffusion and material exchange compared to solid or semi-liquid core counterparts due to their fluidic nature
What this article adds:	• LCM exhibited significantly greater cell proliferation and osteogenic differentiation of encapsulated cells, as evidenced by increased alkaline phosphatase activity and calcium deposition over time. • Semi-LCM showed 2.5-fold greater mechanical strength than LCM, highlighting a trade-off between mechanical stability and cellular performance in bone tissue applications. • LCM effectively overcome limitations associated with traditional cell delivery methods, providing a promising, minimally invasive solution for treating critical bone defects. • Multilayered Core-Shell Microcapsules with Liquid or Semi-Liquid Cores: A Breakthrough in Cell Therapy for Bone Regeneration

1. Introduction

New inspiring strategies are yet to be developed as alternatives to conventional procedures for bone regeneration [1]. As the second most implanted tissue in the body after blood, bone is the major target of many studies in the field of biomedical engineering and regenerative medicine because of its important roles in the body [1, 2]. Wide range of cell sources including both undifferentiated (stem) and differentiated (somatic) cells have been used for bone reconstruction, among which adipose tissue-derived mesenchymal stem cells (AMSCs) are used in this study [3, 4]. It has also been demonstrated that combining AMSCs with a suitable scaffold significantly enhances and accelerates bone regeneration [5].

Several studies have shown that the results of systemic administration or direct cell injection are not desirable mainly because of uncontrolled cell distribution, cell loss due to mechanical stresses or host immune responses and limited control over cell fate after injection [6, 7]. Emerging strategies using multilayer hydrogels and AMSCs with engineered scaffolds, have shown great promise for enhancing bone regeneration through improved osteogenesis, vascularization, and innervation [8, 9]. Hence, cell encapsulation in hydrogel-based compartments to act as a barrier against the mechanical stresses and even host immune response to some extent, is quite essential [10–12]. Plain cell-laden capsules, however, might not be able to effectively block the humoral components of the host immune system such as immunoglobulin G (IgG) [13]. Thus, layer-by-layer (LBL) deposition of oppositely charged polymers, such as alginate and chitosan, was employed to enhance immunoisolation, as evidenced by improved capsule stability and reduced immune recognition [13–16].

Injectable carriers in microscopic scales also have the ability to fill irregularly shaped defects in the body in a minimally invasive manner, increasing patient comfort and reducing the recovery time [17, 18]. These core-shell microcapsules must provide a suitable microenvironment for cell proliferation and function, properly. Core-shell microcapsules create engineered microenvironments that preserve the cells viability and function while providing immune protection via selectively permeable shells. Independent tuning of core and shell layers enables these structures to mimic physiological niches and support scalable and injectable formats ideal for regenerative therapies [19, 20]. Previous studies have shown that the microcapsules with solid cores not only lack the optimal diffusive properties and cause hypoxia, nutrients shortage and toxic metabolic accumulation, but also prevent proper cell migration and cause cell clusters formation. Liquid cores address these issues by enhancing diffusion and cell mobility, as confirmed by improved cell proliferation in this study [21–24].

AMSCs are known to be anchorage dependent and cannot sustain their viability in the liquid cores [3, 25]. Then, adding nano-hydroxy apatite (nHA) particles in the core of the capsules has been proposed and investigated in previous studies. nHA particles not only provide the surface for cell attachment, but also are known for their osteoconduction, osteoinduction and osteointegration effects. nHA-enriched scaffolds significantly enhance bone defect repair by offering high surface area for osteoblast adhesion, and proliferation, strong osteoconductive and osteoinductive effects, and excellent biocompatibility for accelerated tissue regeneration [26,27]. HA is the major component of natural bone extracellular matrix (ECM) in the form of carbonated hydroxyapatite [25, 28–30].

For core preparation, alginate is a suitable candidate in bone tissue engineering, due to its low cost and high mechanical properties [18, 31, 32]. However, its limited cell attachment sites were addressed in this study by incorporating gelatin into the core. The alginate-gelatin blend, combined with nHA, provides an optimal microenvironment for cell adhesion and

proliferation, as validated by MTT assay results [33–35]. This mixed composition of two polymers in the core, could provide both liquid or semi-liquid core for the prepared multi-layered microcapsules using calcium chelation on alginate and/or incubation at 37 °c for gelatin liquefaction [36-38].

2. Materials and Methods

2.1. Materials

Gelatin from porcine skin type A, β-glycerophosphate, ascorbic acid, dexamethasone, alizarin red-S and MTT powder were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium alginate, $CaCl_2$, dimethyl sulfoxide (DMSO), acetic acid, sodium citrate and formalin were prepared from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), high glucose Dulbecco's Modified Eagles Medium (DMEM), phosphate buffer saline (PBS) and trypsin/EDTA were purchased from Bioidea (Tehran, Iran). Chitosan and nHA were purchased from ACROS Organics (Belgium) and penicillin-streptomycin was prepared from Gibco (Waltham, MA, USA).

2.2. Preparation of Cell-free Multilayered Microcapsules with Liquid and Semi-liquid Cores

Preparation of alginate-gelatin microcapsules was according to our previous studies with some improvements [39]. Briefly, the polymer solution containing alginate 1% (w/v), gelatin 5% (w/v) and nHA 0.5% (w/v) in distilled water was extruded (80 mL/hr) through a 23 G needle by syringe pump. The droplets were formed from a co-axial nozzle, using air flow (0.12 bar), into $CaCl_2$ bath (1.47% w/v), stirred for 15 min to crosslink and then, washed using distilled water [15]. For obtaining LBL microcapsules, at first, alginate 0.05% (w/v) and chitosan 0.05% (w/v) solutions in distilled water were separately prepared at pH 6. Then, the obtained microcapsules from previous step were sequentially stirred in the prepared chitosan and alginate solutions, separately, for 3 min and washed for 1 min between each layer deposition to remove the unabsorbed polymers. Eleven layer deposition were performed to provide enough mechanical strength for the multilayered micro-capsules.

Finally, one group containing a half number of the prepared multilayered microcapsules were immersed in 55 mM sodium citrate solution at pH 6 for 1 min to liquefy the calcium alginate part of the cores. Then, they were washed with distilled water and incubated at 37 °C for gelatin liquefaction to produce liquefied microcapsules. The other group containing a half number of the multilayered microcapsules were directly incubated at 37 °C without sodium citrate treatment, to liquefy the gelatin part in the cores only, to produce semi-liquid core microcapsules [37].

2.3. Zeta Potential of the Prepared Cores

Zeta potential analysis was used to determine which polymer (alginate or chitosan) is more suitable as the first layer for deposition on the core of the microcapsules. The prepared cores were suspended in distilled water and investigated by DLS (HORIBA SZ-100) at pHs of 5.5 and 6.5, separately.

2.4. Morphology of the Microcapsules

Overall shape of the microcapsules, were investigated using inverted optical microscope (Nikon, Japan). The pictures were obtained at 40x and 100x magnification, both for the cores (before layer deposition) and after 11 layer deposition, from the liquid and semi-liquid core microcapsules.Scale bars and imaging conditions were standardized to ensure accurate size

2.5. FTIR Analysis

Five samples of the microcapsules, including core only and those containing 1-4 layers, separately, were investigated using FT-IR device (PerkinElmer). The obtained results were analyzed using PerkinElmer Spectrum Version 10.03.06.

2.6. Biochemical Stability of the Cell-free Microcapsules

The sterilized liquefied and semi-liquefied cell-free microcapsules were prepared separately and put (in the specified amount, with 3 replications) in 24-well tissue culture plates. The stability of the microcapsules was investigated in the incubator at 37 °C and 5% CO_2 in DMEM for 21 days. The culture medium of each well were replaced every 3 days and at the end of days 1, 3, 5, 7, 14 and 21, the number of not destroyed microcapsules were counted.

2.7. Mechanical Stability of the Cell-free Microcapsules

Mechanical stability of the microcapsules was evaluated using rotational stress test [40]. Three groups of the multilayered microcapsules were prepared, separately: liquid core (at 37 °C, with sodium citrate treatment), semi-liquid core (at 37 °C, without sodium citrate treatment) and solid core (at 4 °C, without sodium citrate treatment). The microcapsules were centrifuged (with 3 replications) in tubes containing 5 mL DMEM, at 3200 rpm for 40 min. In time intervals of 10 min, the number of not destroyed microcapsules was counted using an optical microscope.

2.8. Cell Encapsulation

AMSCs at the first passage were purchased from Stem Cell Technology Research Center (Tehran, Iran). The obtained cells at second passage were suspended ($5*10^5$ cells/mL) in the prepared polymer solution (alginate 1% (w/v), gelatin 5% (w/v) and nHA 0.5% (w/v) in distilled water) and the cell-laden multilayered microcapsules were prepared according to section 2.2.

2.9. MTT Assay

Metabolic activities of the cells as an indicator of cell viability was measured using MTT assay for three different groups, separately: the non-encapsulated cells in 24-well cell culture plate (as control group), liquefied and semi-liquefied cell-laden microcapsules. The cellular samples were investigated in DMEM (10% FBS) at 37 °C and 5% CO_2, for 7 days. MTT solution (5 mg/mL in PBS) was diluted 4-fold in complete cell culture medium. Viability of the cells (in 3 replications) was assessed at day 0, immediately after cell encapsulation (to investigate the effect of encapsulation process on the cells) and days 3, 5 and 7 as the following: culture medium was completely removed from the wells at the end of each time interval and the prepared MTT solution (0.5 mL) was added to each well. After incubation for 4 h, MTT solution was removed and the formed formazan crystals were dissolved in 0.5 mL of DMSO. Absorbance of the samples was measured by ELISA reader (Ewareness, USA) at 545 nm.

2.10. Alkaline Phosphates (ALP) Activity

ALP activity of the microencapsulated cells was investigated using Biorex Fars kit (Fars, Iran) based on Deutsche Gesellschaft Fur Klinische Chemie (DGKC) method. Four different groups of cell-laden microcapsules, including liquefied and semi-liquefied core microcapsules in osteogenic differentiation medium (ODM) and normal cell culture medium, separately were investigated for 21 days (in 3 replications). At the end of days 7, 14 and 21, the amounts of appeared p-nitrophenol, due to ALP activity, was measured by ELISA reader at 405 nm according to the kit manufacturer's instructions.

2.11. Calcium Deposition Measurement

Formation of calcium phosphate by microencapsulated cells was determined using alizarin red assay. Six groups of multilayered microcapsules were investigated at the end of days 14 and 21 of incubation (in 3 replications): liquefied and semi-liquefied core cell-laden microcapsules in ODM, liquefied and semi-liquefied core cell-laden microcapsules in normal cell culture medium and liquefied and semi-liquefied core cell-free microcapsules. Briefly, at days of 14 and 21, the medium was removed from each well and after rinsing the samples with PBS, the samples were fixed by 4% (v/v) formaldehyde solution at room temperature for 15 min. Then, the fixed cells were stained with 2% alizarin red-S for 15 min at room temperature. Afterwards, the samples were washed with deionized water and observed by optical microscope [41]. For quantification the calcium amount, 500 µL of acetic acid (10% (v/v)) was added to each sample and stirred. Then, the absorbance of 200 µL of each final sample was measured at 405 nm by ELISA reader.

2.12. Statistical analysis

All experiments were performed in triplicate, and the results are reported as mean ± standard deviation.

3. Results and Discussion

3.1. Morphology of the Microcapsules

The spherical and uniform alginate-gelatin-nHA cores of the microcapsules are shown in Figure 1.a. The average size of the produced cores are 500-600 µm. After 11 layers deposition of alginate-chitosan polymers, a clear shell appears between the core and surrounding, as shown in Figure 1.b. This shell not only plays as an important role in maintaining the mechanical strength of the microcapsules, but also may provide higher immunoisolation for the encapsulated cells and reduce the stress and tension on the cells [13, 14]. The multilayered microcapsules showed an average size of 600-650 µm. Once the cores was liquefied, the microcapsule swollen to 850-900 µm and the core spreads uniformly all inside the microcapsule as shown in Figure 1.c. This liquefied core provides suitable diffusive properties and may improve cell migration and proliferation in the microcapsules [22]. The clear shells and uniformity of the prepared microcapsules are shown in Figure 1.d.

The liquefied microcapsules maintained their shape and strength on a dry surface (Figure 2.a). By tearing the shell using a fine needle, the liquid inside the microcapsules spread on the surface (Figure 2.b).

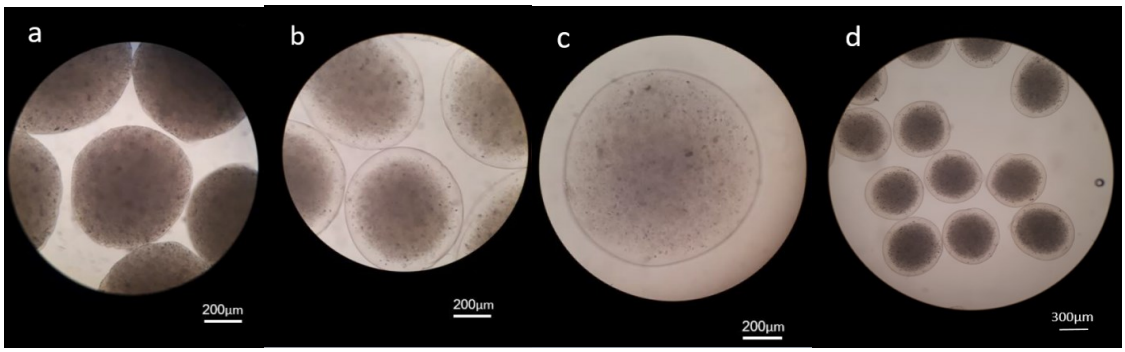

Figure 1. Alg-Gel-nHA cores of the microcapsules (a), eleven layered microcapsules (b), liquefied-core microcapsule (c), and solid-core multilayer microcapsules (d) using optical microscope

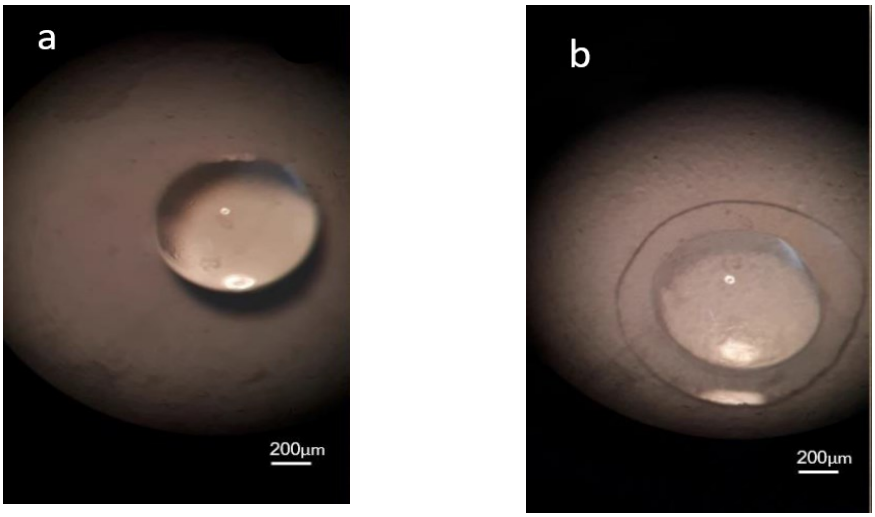

Figure 2. The liquefied multilayered microcapsule on a dry surface (a), and spreading the liquid inside the microcapsule, after tearing using a fine needle (b)

3.2. Zeta Potential Investigation

The obtained results of zeta potential for chitosan, alginate, gelatin and core of the microcapsules have been presented in Figure 3. According to the composition of the cores (gelatin 2.5% and alginate 1%), the related line is closer to the results of pure gelatin. It is also appeared that zeta potential of the core, approximately is between alginate and chitosan lines at pH 6. These results show that in theory, each one of alginate or chitosan may be selected as the first layer on the core. But in practice, alginate was chosen as the first layer in the vicinity of the cellular environment, because of formation of more uniform layer and its perfect biocompatibility. For the next layers, chitosan and alginate were used, alternately.

3.3. FTIR Results

The layer deposition process, was only investigated for 4 layers (1-4), separately. For the first layer of alginate, as shown in Figure 4, the appeared peaks at 1025 and 1075 cm^{-1} represent the stretching vibrations of O-C-O and C-O-C, respectively. There are also intensive peaks at 1424 and 1623 cm^{-1} which are characteristic bands for alginate, related to asymmetric and symmetric COO^- stretching vibration of carboxylate ions [42]. More absorbance in the third layer (alginate), represents more alginate in the structure and confirms deposition of the next alginate layer. For two chitosan layers (layers 2 and 4), the characteristic bands are present at 1035 and 1075 cm^{-1} (C-O stretching vibrations), 1325 and 1375 cm^{-1} (C-H_2 and C-H_3) and 1584 cm^{-1} (bending vibrations of the –NH group derived from amide II) [43]. More absorbance in the fourth layer (chitosan) represents more chitosan in the structure and confirms deposition of the next chitosan layer, as well.

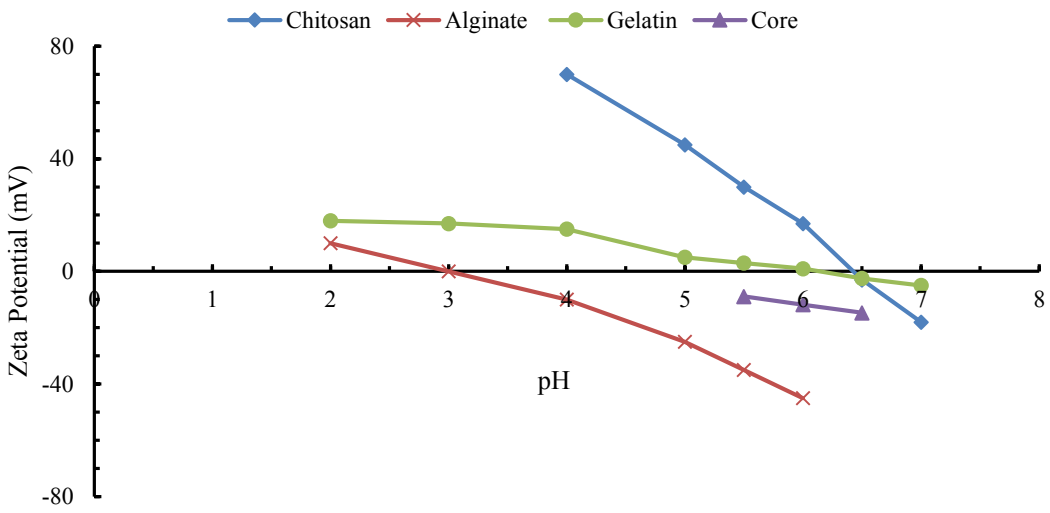

Figure 3. Zeta potential of alginate, gelatin, chitosan and the prepared cores of the microcapsules at different pHs

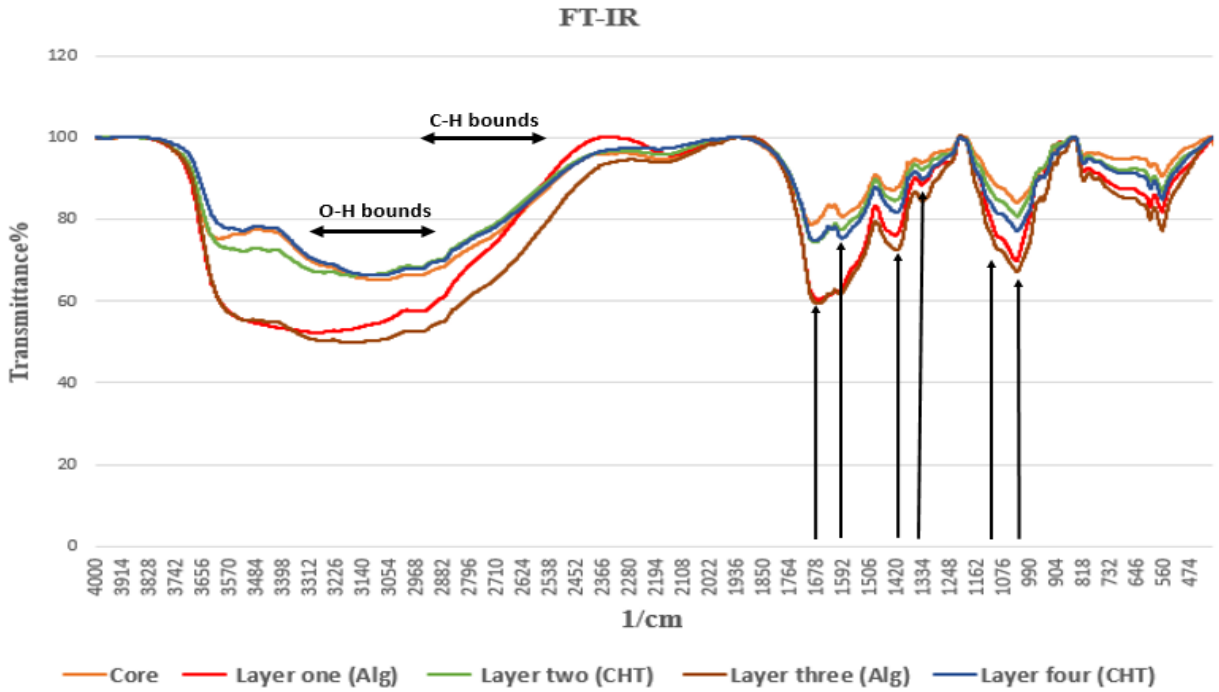

Figure 4. FTIR results of the core (gelatin 2.5% and alginate 1%), and 4 layers deposition (alginate- chitosan- alginate- chitosan, alternately)

3.4. Stability of the Microcapsules

After culturing the prepared microcapsules in DMEM for 21 days, the liquid-core microcapsules showed less stability in comparison to the semi-liquid core microcapsules, as it was expected (Figure 5). At the end of day 21, 9.2±0.59% and 13.5±0.49% of the semi-liquid and liquid core microcapsules, respectively, experienced physical damaging in cell culture medium. Most of the microcapsules damaging seemed to be happened during the cell culture medium exchanging in the liquid core microcapsules.

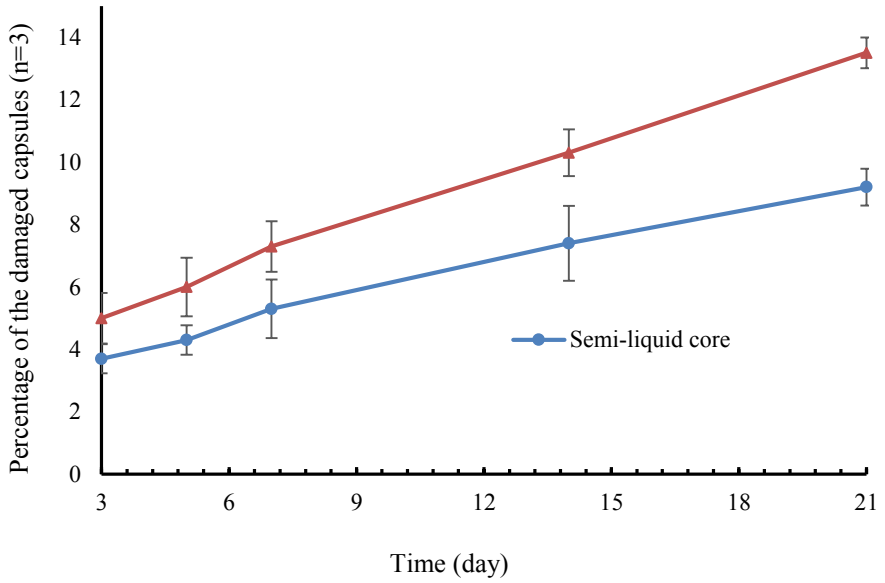

Figure 5. Stability of the liquid and semi-liquid core microcapsules in cell culture medium

3.5.Mechanical Strength of the Micro-capsules

Mechanical stability of the microcapsules was evaluated using rotational stress test. The liquid core microcapsules showed less mechanical strength significantly in comparison to semi-liquid and solid core microcapsules. According to Figure 6, percentage of the damaged microcapsules with liquid core was 2.5–fold higher than the semi-liquid core microcapsules and 1.5–fold higher than the solid core microcapsules. The results showed that percentage of the damaged semi-liquid core microcapsules is less than solid core microcapsules, probably due to the flexible core of semi-liquid microcapsules. They practically undergo physical changes in their shape, become elliptical under stress without any damaging on the shells or core and change back to their original form, slightly after the force is removed. But, solid cores suffer from rigidity and liquid cores lack enough mechanical strength and their shells and cores are ruptured.

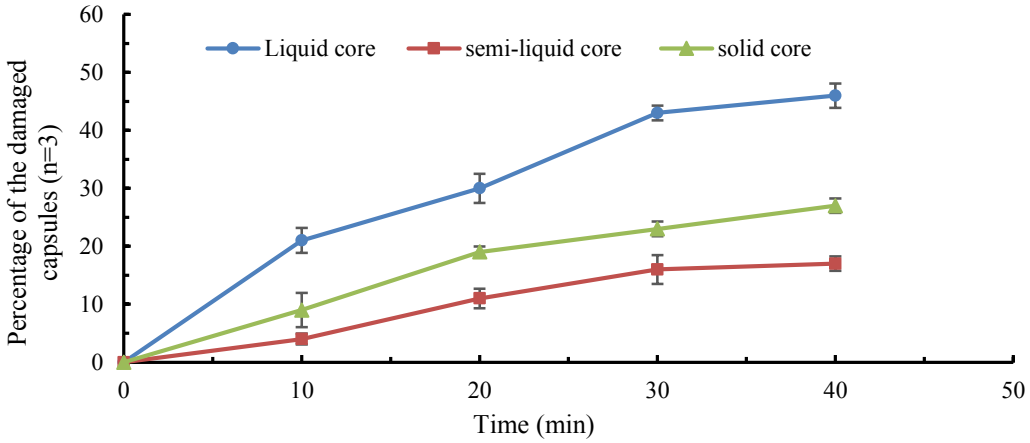

Figure 6. Mechanical strength of the multilayered microcapsules evaluated by rotational stress test

3.6. Cell Proliferation

Encapsulation process of ASCs does not significantly affect their viability. The obtained results from MTT assay at day 0, immediately after encapsulation process, shows negligible decreasing in metabolic activity of the encapsulated cells in both liquid and semi-liquid core microcapsules in comparison to the control group. Metabolic activity of the cells in liquid core microcapsules experienced continuous growth during the time. As it is illustrated in Figure 7, metabolic activity level of the cells in liquid core microcapsules is significantly higher than that one in the semi-liquid core microcapsules and control group. It is may be due to proper diffusive properties and proliferation of the cells in liquid environment in the presence of hydroxyapatite nanoparticles. Semi-liquid core microcapsules and the control group follow the same pattern during the time with slightly higher levels of metabolic activity in the semi-liquid core microcapsule which may be due to 3D environment of the spherical microcapsules that provides more surface area for the cells to attach and proliferate.

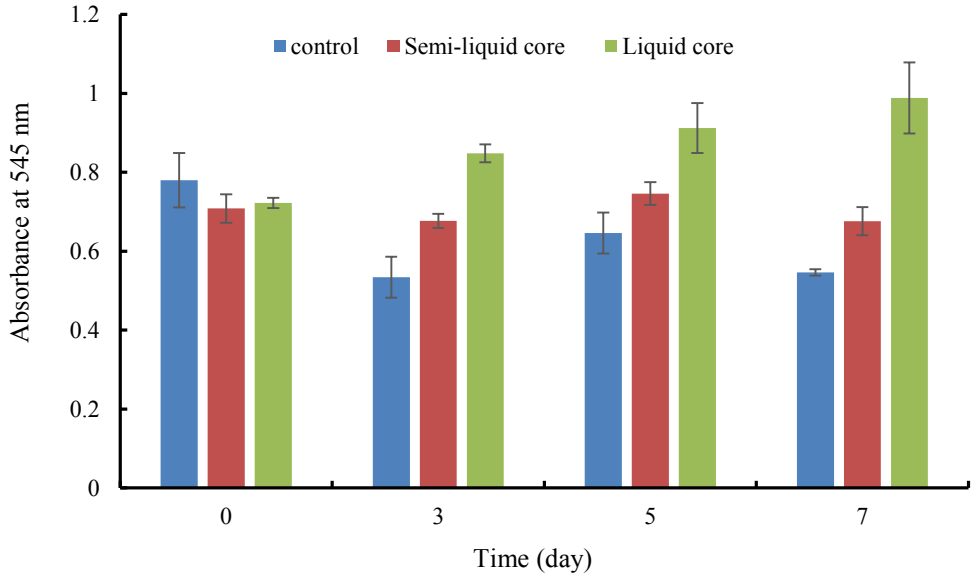

Figure 7. MTT results for liquid and semi-liquid microcapsules at days 0, 3, 5 and 7

3.7. ALP Activity of the Encapsulated Cells

ALP activity of the microencapsulated cells was investigated using Biorex Fars kit. According to the presented results in Figure 8, liquid core microcapsules in ODM at day 7, showed the highest level of ALP activity, approximately 2-fold higher than semi-liquid core microcapsules in ODM and more than 3-fold than control groups. ALP levels of ODM groups decreased during the time which probably shows the beginning of calcification in the differentiated cells [28, 30].

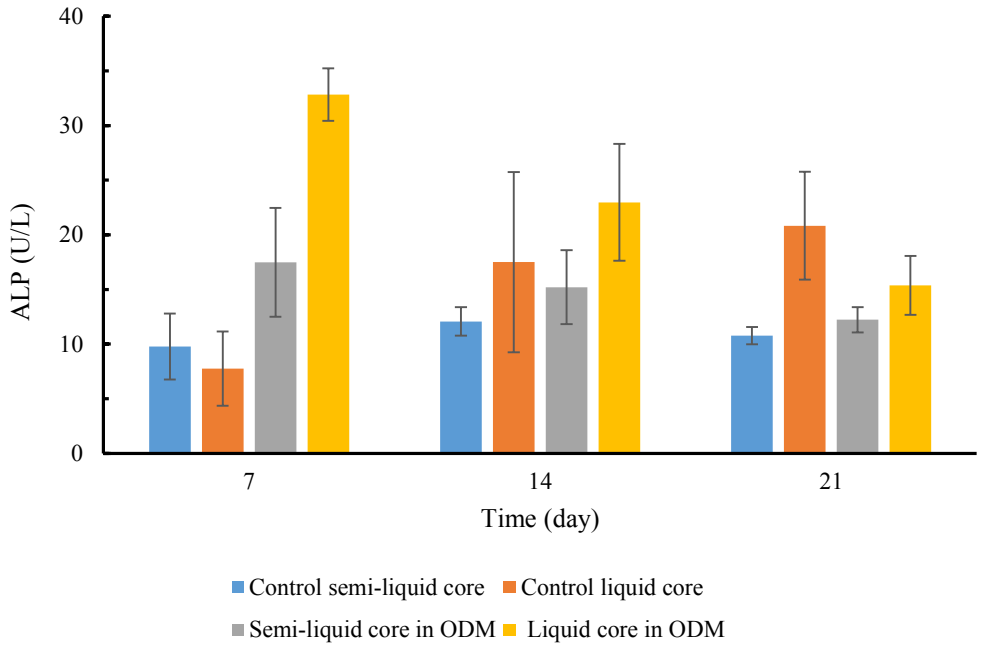

Figure 8. ALP activity of the microencapsulated cells, during 21 days

3.8. Calcium Deposition

Formation of calcium phosphate by the microencapsulated cells was determined using alizarin red assay. Figure 9 shows that cell-laden liquid core microcapsules presented the highest level of calcium deposition at day 21, which is in accordance with the obtained results from MTT assay and ALP activity measurement. All of the investigated cellular groups showed a significant increase in calcium deposition at day 21 in comparison to day 14, except for semi-liquid core microcapsules which didn't experience a noticeable increase, as shown in Figure 9. Cell-free microcapsules also presented the same and lowest amounts of calcium deposition at days 14 and 21, probably due to the presence of calcium in the structure of nHA and as the crosslinking agent of sodium alginate in the cores.

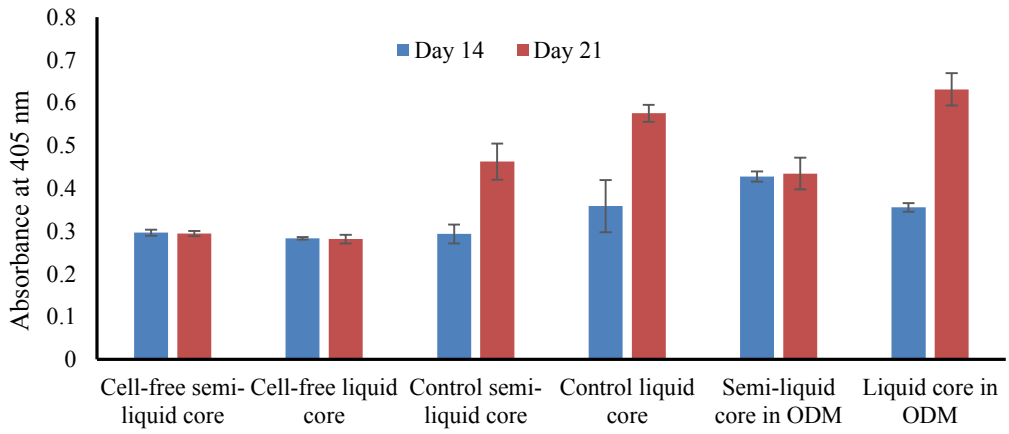

Figure 9. Quantification of calcium deposition in the microcapsules using alizarin red staining, during 21 days

The pictures of different stained microcapsules are presented in Figure 10. The cell-free microcapsules, as shown in Figure 10.a, show light red to orange color in the core, which is due to calcium presence in the structure of nHA or calcium used in crosslinking process. Figure 10.b, shows the cell-laden microcapsules in ODM after staining, which has a dense and dark red color in the core. The cell-laden microcapsules in normal cell culture medium are shown in Figure 10.c, and finally a group of cell-laden microcapsules in ODM are presented in Figure 10.d, with a different magnification. All the pictures are taken at day 21 and perfect spherical shape of the microcapsules and their shells have been lost due to multiple washing stages in the staining procedure.

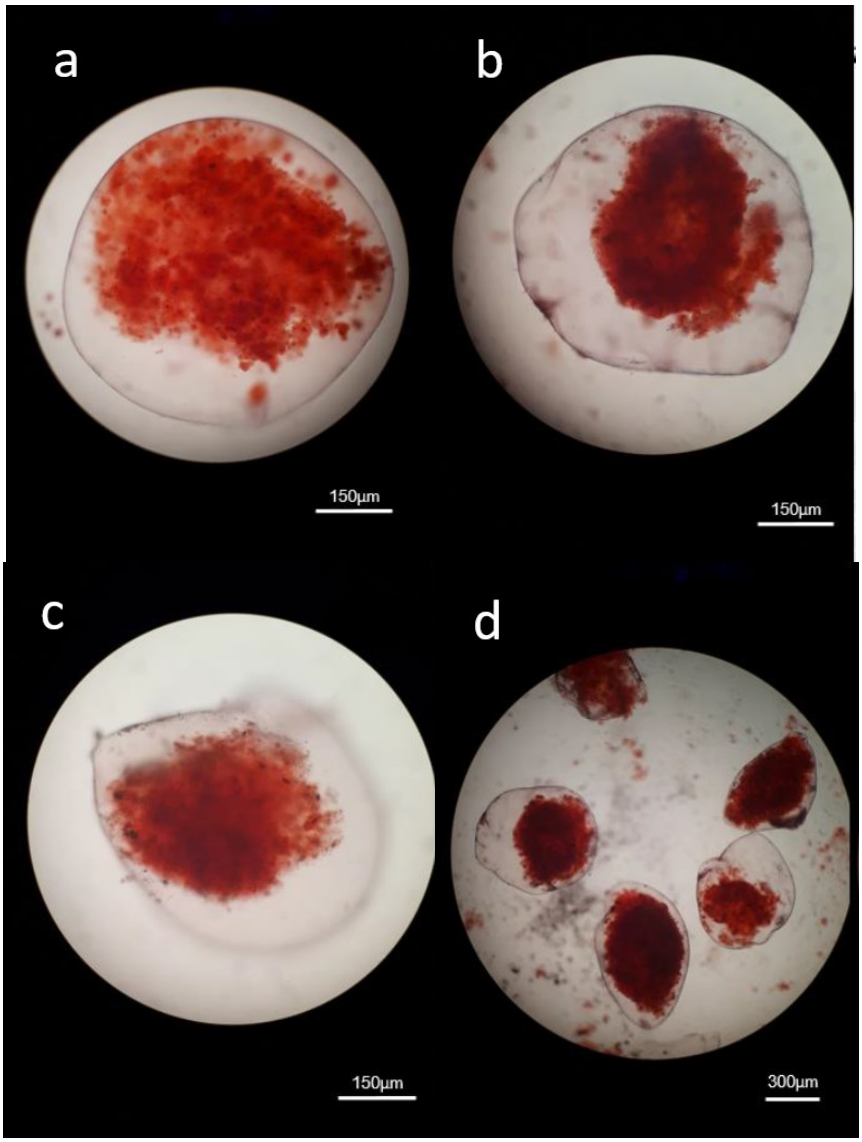

Figure 10. Alizarin red stained microcapsules: cell-free microcapsule (a), cell-laden microcapsule in ODM (b), cell-laden microcapsule in cell culture medium (c), and cell-laden microcapsules in ODM (d), at day 21

4. Conclusion

Multilayered microcapsules represent a promising platform for cell therapy and drug delivery. The alginate-gelatin-nHA core, combined with LBL-deposited alginate-chitosan shells, provides robust mechanical support and immunoisolation. Liquid-core microcapsules demonstrated superior cell

proliferation and osteogenic differentiation due to enhanced diffusive properties, despite lower mechanical stability compared to semi-liquid cores. Future studies should explore reinforcing liquid-core microcapsules (e.g., via additional layers or polymer modifications) to address mechanical limitations. This approach offers a minimally invasive, effective solution for filling critical bone defects.

5. Declarations

5.1. Acknowledgments

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5.2. Authors'Contributions

Conceptualization: Sameereh Hashemi-Najafabadi, Ebrahim Vasheghani-Farahani; methodology: Keyvan Abedi Dorcheh; formal analysis and investigation: Keyvan Abedi Dorcheh; writing—original draft preparation: Keyvan Abedi Dorcheh; writing—review and editing: Keyvan Abedi Dorcheh, Sameereh Hashemi-Najafabadi, Alireza Zarei-Dastgerdi**;** funding acquisition: Sameereh Hashemi-Najafabadi; supervision: Sameereh Hashemi-Najafabadi. All authors have read and agreed to the published version of the manuscript.

5.3. Declaration of Interest

The authors of this article declared no conflict of interest.

5.4. Ethical Considerations

All ethical principles were adhered in conducting and writing this article.

5.5. Transparency of Data

In accordance with the principles of transparency and open research, we declare that all data and materials used in this study are available upon request.

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