TISSUE ENGINEERING CONSTRUCTS AND CELL SUBSTRATES

Original Research

Cell laden alginate-keratin based composite microcapsules containing bioactive glass for tissue engineering applications

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Abstract

Microcapsules based on alginate-keratin, alginate dialdehyde (ADA)-keratin and ADA-keratin-45S5 bioactive glass (BG) were successfully prepared. The samples were characterized by light microscopy, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The results showed that ADA-based materials possess higher degradation rate compared to alginate–based materials. The incorporation of BG particles (mean particle size: 2.0 μm) improved the bioactivity of the materials. Moreover, the biological properties of the samples were evaluated by encapsulating MG-63 osteosarcoma cells into the microcapsules. The cell viability in all samples increased during 21 days of cultivation. However, the presence of 0.5% BG particle seemed to have initial negative effect on cell growth compared to other samples without BG. On the other hand, the positive effect of CaP formation was visible after 3 weeks in the BG containing samples. The results are relevant to consider the development of cell laden bioinks incorporating inorganic bioactive particles for biofabrication approaches.

Graphical Abstract
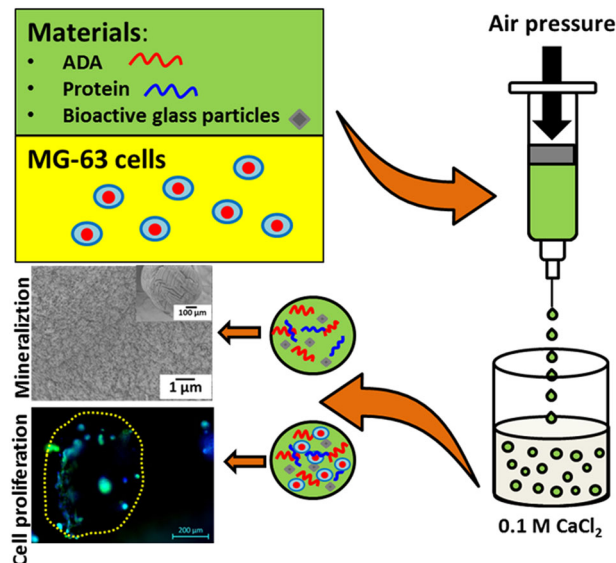
1 Introduction

Tissue engineering has emerged as an alternative approach for the repair and regeneration of damaged tissues and organs [1]. Several approaches have been put forward for the development of scaffolds for tissue engineering with more recent efforts focusing on the development of suitable

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hydrogels for encapsulating cells and for biofabrication of complex tissue-like constructs [2–4]. 3D cell encapsulation in hydrogels attracts increasing attention for tissue engineering applications because hydrogels provide a hydrated matrix closely mimicking the in vivo environment for cell and tissue growth [5]. Moreover, hydrogel matrices can protect transplanted cells from an immune response and they prevent cells from migrating away from the target site [6, 7]. Alginates are a family of polysaccharides consisting of (1, 4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues [8]. Alginate-based hydrogels are being widely used in tissue engineering due to their biocompatible character and their structural features similar to the extra cellular matrix [9]. Because of its gentle gelation process in the presence of divalent cations such as Ca^{2+} and Ba^{2+}, cell encapsulation is one of the popular applications of alginate in the field of tissue engineering [10]. However, alginate hydrogels degrade very slowly and exhibit poor cell adhesion capability because of their lack of specific biomolecular anchoring sites for mammalian cells [11]. To overcome these drawbacks of alginate, several approaches are being investigated. For example, the oxidation of alginate leading to alginate dialdehyde (ADA) can enhance its biodegradability [9], and the incorporation of specific proteins into alginate based hydrogels can improve the cell-matrix interaction of the hydrogel [12, 13]. The oxidized uronate residues make ADA more susceptible to alkali catalyzed elimination resulting in faster biodegradability [9]. Keratin is a protein found as a main component in skin, hair, nail, hooves and horns. Keratins extracted from human hair and wool contain cell adhesive peptide sequences including RGD (arginine-glycine-aspartic acid), and LDV (leucine-aspartic acid-valine) [14, 15]. The combination of alginate and keratin has been exploited only to a limited extent to develop tissue engineering scaffolds or for drug delivery vehicles [14, 16, 17] in comparison to ADA-gelatine combinations [18–20].

Bioactive glasses (BGs) are well known for promoting calcium phosphate (or hydroxyapatite) deposition when in contact with physiological fluids [21–23], which is favorable for bone regeneration applications [21]. Cell incorporation in BG-polymer matrices (composite hydrogels) for bone tissue engineering using different types of hydrogels such as alginate, silk fibroin-gelatin, ADA-gelatin, and elastin-like polypeptide-collagen have been reported [7, 22, 24–26]. The present study considers for the first time the fabrication of alginate-keratin based composite microcapsules containing BG particles of 45S5 BG composition [21] for cell encapsulation. We propose the use of ADA synthesized via periodate oxidation of alginate and keratin extracted from wool fabric. Unoxidized alginate-keratin was used as a control. The hypothesis was that introducing BG particles into the microcapsules would enhance the

biomineralization capability of the matrix to promote osseointegration, which would confirm the application potential of the new microcapsules in bone tissue engineering.

2 Materials and methods

2.1 Hydrogels

2.1.1 ADA synthesis

ADA was produced according to Zehnder et al. [27] by oxidizing sodium alginate (alginic acid sodium salt from brown algae, suitable for immobilization of microorganisms, MW 100000-200000g/mol, Sigma-Aldrich, Germany) in an ethanol-water mixture system using sodium metaperiodate (VWR Int.) as an oxidant. An amount (10 g) of alginate was dispersed in 50 ml ethanol and 2.674 g of sodium metaperiodate were dissolved in 50 ml ultrapure water. The periodate solution was slowly added to the sodium alginate solution under continuous stirring in a dark environment at room temperature for 6 h. The reaction was quenched by adding 10 ml ethylene glycol under continuous stirring for 30 min. The resultant suspension was dialyzed against ultrapure water for 5 days with 10 changes of water until the dialysate was periodate free. The absence of periodate was checked by adding a 0.5 ml aliquot of the dialysate to 0.5 ml of a 1% (w/v) solution of silver nitrate. The ADA solution was then frozen and lyophilized.

2.1.2 Keratin extraction

Delipidated wool fabric was used as a source of keratin for keratin extraction. 1% keratin solution was prepared as reported by Silva et al. [14]. Briefly, 1 g of delipidated wool fabric was immersed in 10 ml of solution containing 8 M urea, 0.2 M sodium dodecyl sulphate (SDS) and 0.5 M $Na_2S_2O_5$. The mixture was incubated at $60^{\circ}C$ for 17 h and diluted with 90 ml of ultrapure water. The solution was then filtered and dialyzed against ultrapure water using dialysis tube (MWCO: 12–14 kDa, Spectrum Lab, USA) for 3 days with several changes of water. Protein content in keratin extract was measured by the Lowry method using bovine serum albumin as a standard [28]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to characterize the keratin extract solution by using the OmniPAGE TETRAD Package (Cleaver Scientific Ltd.). The resolving gels (10% acrylamide of about 1.5 mm thickness) were run at a constant voltage (120 V) and prepared according to the method described by Laemmli [29]. Proteins were visualized by Coomassie Brilliant Blue G
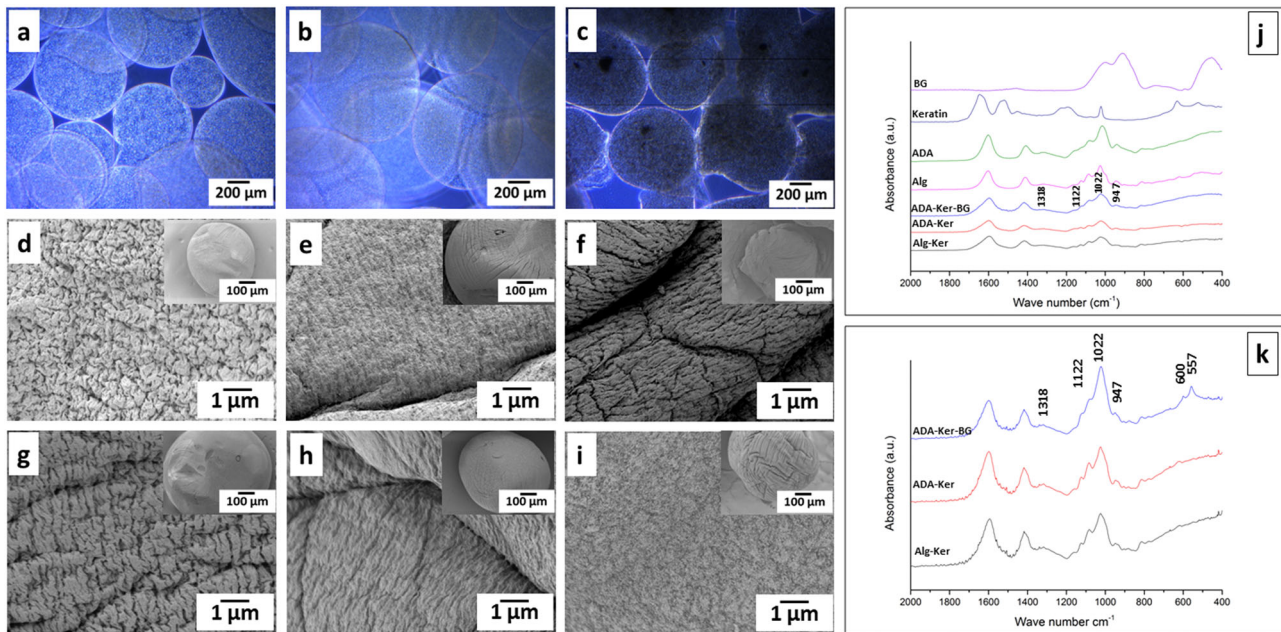
Fig. 1 Light microscopy images of microcapsules produced from **a** Alg-Ker, **b** ADA-Ker, **c** ADA-Ker-BG, SEM images of microcapsules fabricated from **d** Alg-Ker, **e** ADA-Ker, **f** ADA-Ker-BG before incubation in SBF, SEM images of microcapsules fabricated from **g** Alg-Ker, **h** ADA-Ker, **i** ADA-Ker-BG after 7 days of incubation in SBF, and FTIR analysis of the samples **j** before and **k** after 7 days of incubation in SBF. (The relevant peaks in the FTIR spectra are discussed in the text)

250 staining using the Prestained Page Ruler marker (Thermo Scientific) for calibration.

2.2 Preparation of hydrogels and microcapsules

Three types of hydrogels were used in this study: (1) alginate-keratin, (2) ADA-keratin, and (3) ADA-keratin-BG. 1%alginate-0.5%keratin which was previously reported by Silva et al. [14] was used as a control. The concentration of keratin was also kept at 0.5% in other samples. In preliminary studies, it was found that when the ADA content in ADA-keratin hydrogel is lower than 2.5%, the material cannot form capsules after the crosslinking process. Therefore, the concentration of ADA in the ADA based hydrogel was kept as 2.5%. To prepare hydrogels, alginate and ADA were dissolved in phosphate buffer saline (PBS; Gibco) to obtain the final concentration of 2% (w/v) alginate and 5%(w/v) ADA. Both alginate and ADA solutions were sterilized using 0.45 μm filters and the 1% keratin solution was filtered through 0.22 μm filters (Roth). 45S5 BG powder (particle size 2.0 μm) with the composition (wt. %): 45% SiO_2-24.5% CaO-24.5% Na_2O-6% P_2O_5 (Schott AG) was heated at 160°C for 2 h to eliminate impurities. To prepare alginate-keratin or ADA-keratin hydrogels, equal volume of 1% keratin solution was mixed with 2% alginate solution or 5% ADA solution. In case of samples containing BG, the sterilized BG powder was dispersed in the keratin solution before mixing with ADA solution to obtain the final concentration of BG at 0.5% (w/v). The prepared

hydrogels were transferred into an extrusion cartridge (Nordson EFD) connected to a high precision fluid dispenser (Ultimus V, Nordson EFD). Microcapsules produced by applying air pressures (1–2 bars) were collected and kept for 10 minutes in 0.1 M $CaCl_2$ solution to allow ionic crosslinking. The microcapsules were then sieved and washed 3 times with Hank's balanced salt solution (HBSS).

2.3 Characterization

Degradation of the fabricated microcapsules was examined by immersing weighed samples (500 ± 20 mg, W_i) in 4 ml of Hank's balanced salt solution (HBSS) at 37°C with controlled atmosphere of 5% CO_2 and 95% relative humidity. The weight of the samples was measured at specific time intervals (W_t). The degradation of the microcapsules was calculated as follows:

$$Degradation \left(\% \, weight \, loss\right) = \frac{(Wi - Wt)}{Wi} \times 100$$

Microcapsule morphology was assessed by bright field microscopy (Primo Vert, Carl Zeiss) and scanning electron microscopy (SEM, Auriga Zeiss). Prior to SEM analysis, the samples were dried using a critical point dryer (Leica EM CPD 300) and coated with gold using a sputter coater (Q150T, Quorum Technologies). FTIR spectrometer (IRAffinity-1S, Shimadzu) was used to evaluate the chemical composition of the samples. The dried samples were used to record attenuated total reflectance Fourier transform

infrared spectroscopy (ATR-FTIR). In vitro bioactivity tests were carried out by incubating samples in simulated body fluid (SBF) [30] at $37\,^{\circ}C$ in 5% CO_2 and 95% relative humidity for 7 days. SBF was changed 3 times a week to avoid any pH changes [31].

2.4 Cell encapsulation

Osteoblast-like MG-63 cells (Sigma-Aldrich, product no. 86051601-1VL) were mixed with hydrogels at the concentration of 10^6 cells/ml of hydrogel followed by the fabrication of microcapsules using the procedure described in Section 2.2. The weighed microcapsules (150 ± 10 mg) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich). Six replicates of samples were used in this study. The viability of the encapsulated cells was measured using WST-8 assay kit (Sigma-Aldrich) following the manufacturer's protocol after 7, 14 and 21 days of cultivation. The live cells in the microcapsules and the nuclei of the cells were visualized using calcein AM (Invitrogen) and blue nucleic acid stain, DAPI (Invitrogen), respectively.

2.5 Statistical analyses

Statistical analyses were performed by one-way analysis of variance (ANOVA).

3 Results and discussion

3.1 Physicochemical characterization

In this study, microcapsules of alginate-keratin (Alg-Ker), ADA-keratin (ADA-Ker) and ADA-keratin-BG (ADA-Ker-BG) were fabricated by a pneumatic extrusion technique. Figure 1(a–c) shows the morphology of different microcapsules. Alg-Ker and ADA-Ker microcapsules have mostly spherical shape with smooth surface, whereas ADA-Ker-BG microcapsules have an irregular shape. This result can be explained by the presence of BG particles which prevented the formation of a continuous matrix and changed the flowing behavior of the mixture during the extrusion process. Regarding the surface morphology of microcapsules (Fig. 1d-f), it was observed that Alg-Ker microcapsules exhibited a regular patterned folded surface. The surface becomes smoother and less folded for the microcapsules containing ADA because of partial oxidation, as explained in previous studies [11]. There is no difference in the surface structure of ADA-Ker and ADA-Ker-BG. This observation indicates that BG particles are covered with the polymeric components of the microcapsules. Before SBF
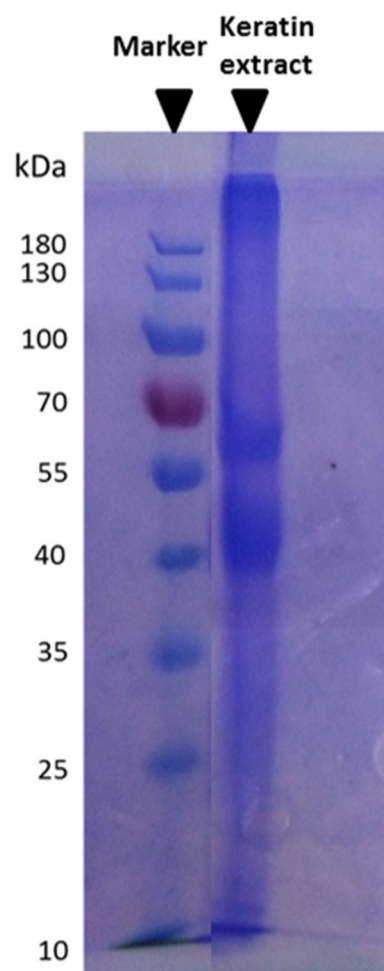

Fig. 2 SDS–PAGE of keratin extract after filtration through $0.22\,\mu m$ filters used in this study

immersion, the FTIR spectra of all samples (Fig. 1j) exhibited the characteristic absorption bands of polysaccharide, e.g. at 1318 and $947\,cm^{-1}$ (C-O stretching), $1122\,cm^{-1}$ (C-C stretching) and $1024\,cm^{-1}$ (C-O-C stretching) [32]. However, the peaks corresponding to amide were not detected. The absence of these peaks might be attributed to the presence of low amount of keratin in the samples. The content of protein in the keratin extract according to Lowry measurements was 0.65% (W/V) and it decreased to 0.47% (W/V) after filtration through 0.22-μm filters. This result indicated that protein in the keratin extract was partially removed during the filtration process. The protein remaining in the keratin extract was characterized by SDS-PAGE electrophoresis in order to analyze its molecular weight. The main bands of the keratin extract were about 40 and 60 kDa, as shown in Fig. 2. These bands are similar to the characteristic bands of low-sulphur content keratin protein (60–45 kDa) previously reported [33]. The fact that keratin was present in the samples was assessed by determining the release in HBSS by the Lowry

method during the degradation study (data not shown), which confirmed the presence of keratin in the samples. No peak related to BG is observed in the spectrum of ADA-Ker-BG capsules. This is in agreement with the morphology of the microcapsules showing no BG particle exposed on
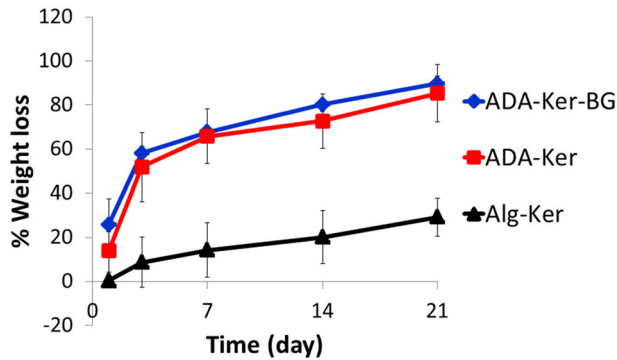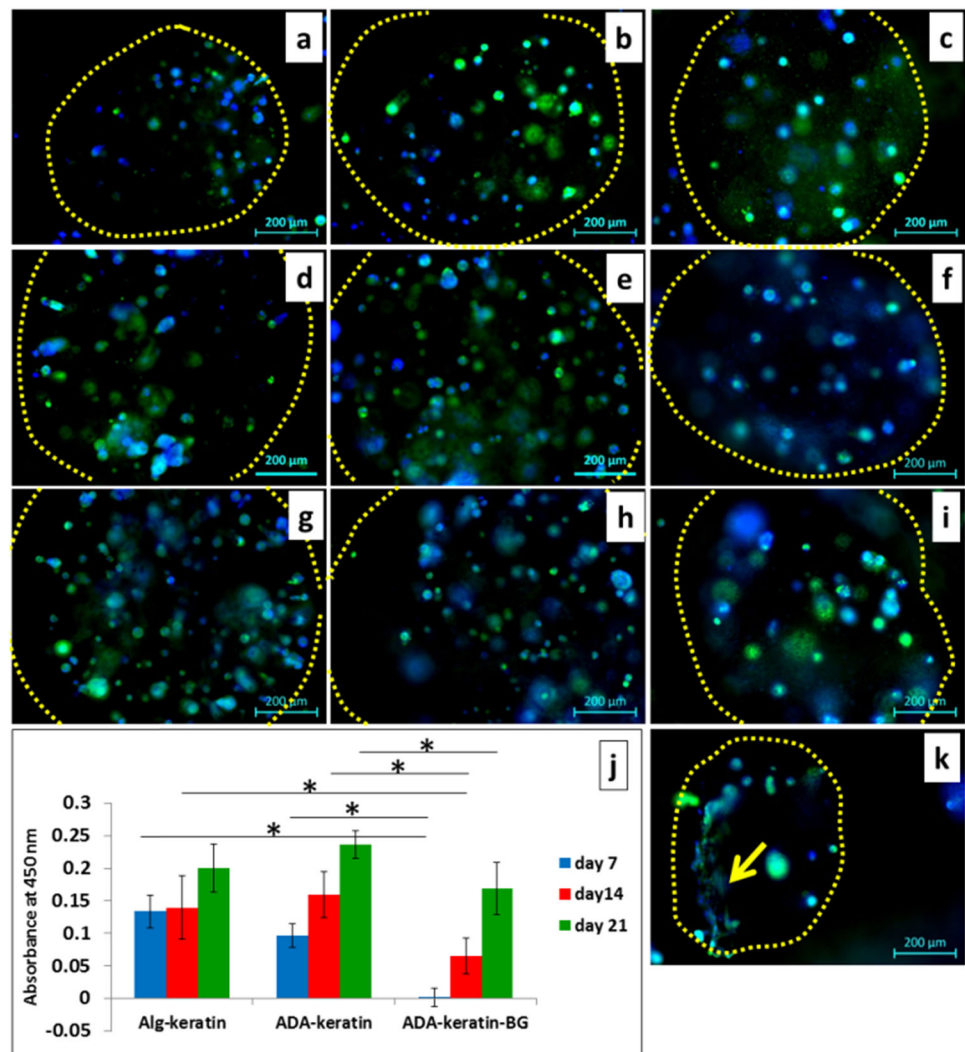

Fig. 3 Weight loss of microcapsules in HBSS showing the faster degradation of ADA based materials compared to the alginate based material

the surface. After incubation in SBF for 7 days, the surfaces of Alg-Ker and ADA-Ker (Fig. 1g, h) show similar structure compared with samples before SBF immersion while deposited particles on the surface of ADA-Ker-BG were observed (Fig. 1i). FTIR spectra of Alg-Ker and ADA-Ker after 7 days in SBF are not different from the respective spectra before SBF immersion while double peaks at ~560 and ~600 cm^{-1} were clearly observed in ADA-Ker-BG samples (Fig. 1k). These two bands could be assigned to the P-O bond in crystallized calcium phosphate, which indicates the occurrence of biomineralization, e.g. the formation of a CaP phase, which is desired for applications in bone tissue engineering [34, 35]. Alg-Ker exhibited a considerably lower degradation profile as compared to the ADA-based samples (Fig. 3). This is because ADA possesses lower molecular weight compared to alginate. Moreover, the oxidized residues are very susceptible to alkaline β-elimination, which enhances the degradation property of ADA [36]. ADA-Ker and ADA-Ker-BG exhibit very similar

Fig. 4 Fluorescence microscopic images of MG-63 cells encapsulated in Alg-Ker (first column), ADA-Ker (second column), ADA-Ker-BG (third column) microspheres after **a–c** 7, **d–f** 14, and (**g–i** and **k**) 21 days of incubation, cell viability of encapsulated MG-63 cells after 21 days of cultivation **j**. Statistically significant differences are indicated as: *p < 0.05 (Bonferroni's posthoc test)

degradation patterns. It is thus likely that the incorporation of BG at the concentration investigated in this study does not change the degradation behavior of ADA-Ker.

3.2 Cell culture study

The cell viability of encapsulated MG-63 cells in all samples tends to increase during the 21 days of cultivation (Fig. 4j). There is no significant difference in cell viability in Alg-Ker and ADA-Ker at each time point. This result is consistent with that of fluorescent analyses illustrating that cells were able to grow after 3 weeks of encapsulation in ADA-Ker and Alg-Ker (Fig. 4a-i). Cells grown in microcapsules showed round cell bodies. However, cells immobilized in ADA-Ker-BG exhibit a significantly lower cell viability compared to other samples without BG. Our findings indicated that the presence of BG particles in ADA-Ker hydrogel with the concentration used in this study initially reduces the viability of MG-63 cells. This is in agreement with the results reported by Rottensteiner et al. [37] that the addition of 45S5 BG nanoparticles (nBG) into ADA-gelatin has slight cytotoxicity to bone-marrow derived mesenchymal stem cells (MSCs) cultured on the hydrogel films. However, Leite et al. [22] found that the viability of MG-63 cells encapsulated in printed scaffolds made from ADA-gelatin containing sol-gel derived bioactive glass nanoparticles was similar to that in samples without nBG. After 21 days of incubation, the cell viability in ADA-Ker-BG capsules becomes comparable to that of Alg-Ker. Interestingly, the spreading phenotype of the cells was noticed on the surface of some ADA-Ker-BG microcapsules on day 21 (Fig. 4k). This fact was not observed in other samples, indicating a possible positive effect of the CaP formation induced by the dissolution of BG particles after 3 weeks.

4 Conclusions

Alginate-keratin based composite microcapsules containing bioactive glass of 45S5 composition were prepared via a pressure-driven extrusion technique. The degradation rate of the samples containing ADA was higher than that of the samples containing pristine alginate. SEM and FTIR analyses suggested that BG particle loading promoted the growth of calcium phosphate on the surface when the microcapsules were immersed in SBF. The viability of MG-63 cells encapsulated in all samples increased over 21 days. Even if the cell viability in samples containing BG was lower than that of other samples in the first 2 weeks, there was no significant difference in cell viability in ADA-Ker-BG and Alg-Ker microcapsules on day 21. The results proved that the developed novel composite hydrogel is a promising material for biofabrication in tissue engineering

such as in bone-tendon interface regeneration. Further studies should consider other types of BGs, for example, nanoparticles or BG compositions incorporating osteogenic and angiogenic ions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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