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A novel nano-composite scaffold for cartilage tissue engineering

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Abstract. In this study, hybrid Poly (lactic-co-glycolic acid) (PLGA)/Hyaluronic Acid (Ha)/Fibrin/45S Bioactive Glass (45SBG) nanocomposite scaffolds seeded with human Adipose-Derived Mesenchymal Stem Cells (hADMSCs) were investigated as a construct for osteoarthritis (OA), Articular Cartilage (AC), and subchondral bone defects therapies. The bioactivity and biodegradation of the nanocomposite scaffolds were assessed in Simulated Body Fluid (SBF) and Phosphate Buffer Saline (PBS) solution, respectively. Furthermore, MTT analysis was performed in order to determine attachment and viability of hADMSCs. Ultimately, results indicate the increase of bioactivity in nanocomposite scaffolds, as compared to the pure PLGA scaffold. In addition, biodegradation assay exhibits that the addition of Ha, fibrin, and 45SBG nanoparticles could modify the degradation rate of PLGA. The nanocomposite scaffolds did not exhibit any cytotoxicity, and the hADMSCs were attached to the scaffolds , which proliferate properly. According to our investigation, it was concluded that using natural and synthetic polymers along with BG nanoparticles may provide a suitable construct and could show a beneficial role in AC tissue engineering and OA therapy.

1. Introduction

Osteoarthritis (OA) is a rampant disease in industrial societies imposing more than $30 billion a year to their therapeutic budget [1]. Articular Cartilage (AC) lesions have a confined capacity to reform following injury, trauma, and pathological diseases [2]. Numerous treatment strategies have been utilized to repair traumatic cartilage defects; however, in these techniques, fibrocartilage formation, immune rejection, pathogen transmission, donor site morbidity, and slower remodelling is not pending away [1-3]. Tissue Engineering (TE) and regenerative medicine are new approaches that can promote cartilage regeneration and transcend the previous methods limitations [4]. TE with three basic elements, including cells, biodegradable scaffolds, and growth factors, strives to compensate the performance of the damaged tissues and organs [5,6]. The scaffolds have a pivotal role in regeneration of tissues and provide simulated environment similar to the Extra Cellular Matrix (ECM) for cell migration, adhesion, and proliferation. In order to design a

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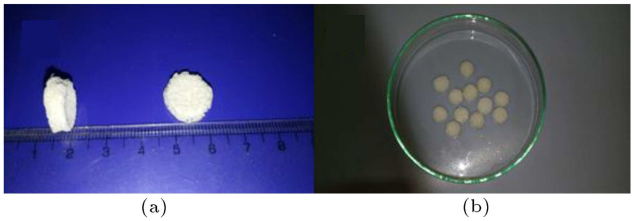
successful structure for regeneration of cartilage tissue disorders, significant parameters include biocompatibility, biodegradability, interconnected porous structure, sufficient mechanical properties, adequate ability to cell attachment, proliferation, differentiation, non-cytotoxicity, and non-antigenicity must be considered [7]. Native biological materials (e.g., hyaluronic acid (Ha), fibrin, etc.) and synthetic polymeric materials are the two biggest groups of material suitable for forming scaffolds [8]. Poly (Lactic-co-Glycolic Acid) (PLGA) is an approved synthetic polymer material by the US Food and Drug Administration (FDA) due to its biocompatibility, biodegradability, facile biodegradation, and good mechanical properties. With its plausible biocompatibility property, PLGA is widely used in bone and cartilage TE applications. However, limitation on cell adhesion and high price are two drawbacks of PLGA as a biomaterial [6-9]. Despite low cell attachment affinity of PLGA, using other materials, such as bioceramics (e.g., Bioactive Glasses (BGs) nanoparticle) as fillers/reinforcement components in PLGA matrix, could provide a distinguished surface in order to improve cellular interaction and intensify its application in hard tissue regeneration [10-12]. Hyaluronic acid (Ha) is a kind of native biological materials that can be found in synovial fluid with excellent ability to support chondrocytes and Mesenchymal Stem Cells (MSCs), noticeable biocompatibility, biodegradability, differentiated cell phenotype, well cell expansion, and little toxicity that can be used in soft and hard tissues [6,13]. Researchers have exhibited that the hybrid scaffolds, containing Ha and the other natural materials (e.g., collagen and fibrin), can restore AC performance and are an effective way for AC rehabilitation [14,15]. Fibrin is a blood derivative that was widely used in clinical operations as fibrin glue. From a cell delivery behaviour's point of view, fibrin has been utilized with Mesenchymal Stem Cells (MSCs) for soft and hard tissues (e.g., ligament, bone and cartilage). Meanwhile, because it suffers from weak mechanical properties and short biodegradation time, TE applications have been limited [6,16,17]. Fibrin application for immobilizing cells and providing homogenous cells distribution in PLGA scaffolds have displayed chondrocytes' proliferation, high level of cartilage-specific proteins secretion and promotion, and the cartilaginous tissue formation [18]. In recent years, researchers have fabricated hybrid nanocomposite scaffolds from synthetically and naturally derived polymeric materials due to high regeneration potential in the context of regenerative medicine [10,19]. Nanocomposite scaffolds are a nano-scale version of a conventional composite, where nanoparticles are dispersed in a polymer matrix [20]. Recently, synthesized Bioactive Glass (BG) nanoparticles, such as 45S, with high biological activity, have been widely used as a filler or coating with polymers in order to regenerate tissues such as bone, lungs, cartilage, and other organs [21]. Previous researches have demonstrated the eminent role of BGs in subchondral and cartilage regeneration [22-25]. Until now, several techniques have been used to confect porous scaffolds, such as Thermal-Induced Phase Separation (TIPS), electrospinning, gas foaming, solvent casting and particulate leaching (SC/PL), and some combined or modified processes of the above [10,26]. The objective of this study, accordingly, is to prepare nanocomposite scaffolds containing PLGA, Ha, fibrin, and 45SBG nanoparticles with SC/ PL techniques and assessment of biological behaviour of hADMSCs on the surface of these scaffolds as a candidate for cartilage TE applications. Likewise, a correlation among Ha, fibrin, and 45SBG nanoparticles' contents will be ascertained.

2. Materials and methods

2.1. Nanocomposite porous scaffolds fabrication

Nanocomposite scaffolds with different amounts of Ha, fibrin, and 45SBG nanoparticles were made via SC/PL techniques, according to our previous work elsewhere [10]. Briefly, PLGA powder (RESOMER® RG 504H, PLGA; 48/52 wt% poly (lactide)/poly (glycolide) and inherent viscosity 0.45-0.60 dl/g ([25°C; 0.1% in chloroform]) were completely dissolved in dichloromethane (CH_2Cl_2, M = 84.93 g/mol) with a concentration of 8% W/V. Afterwards, sodium chloride salt particles (NaCl), as porogen, were added to cylindrical silicon moulds (9 mm in diameter and 3 mm in height), and polymer/solvent solution was cast into them. Figure 1(a) and (b) showed the mould shape and macroscopic picture of the pure PLGA scaffold, respectively. Moreover, Figure 2 shows TEM image of 45SBG nanoparticles (particle size $\leq$ 150 nm).

Then, the samples were air-dried for 2 days and immersed in deionized water for 72 h in order to leach out porogen particles. At the end, a freeze-drying process was performed at -80°C for 2 days and, then, samples were stored under vacuum condition. The porosity percentage and the pore size of the PLGA scaffold were 87.01 ± 03 and 100-200 μm, respectively. Nanocomposite scaffolds were prepared by coating



(a) (b)

Figure 1. (a) and (b) Macroscopic images of the scaffolds prepared via SC/ PL techniques.

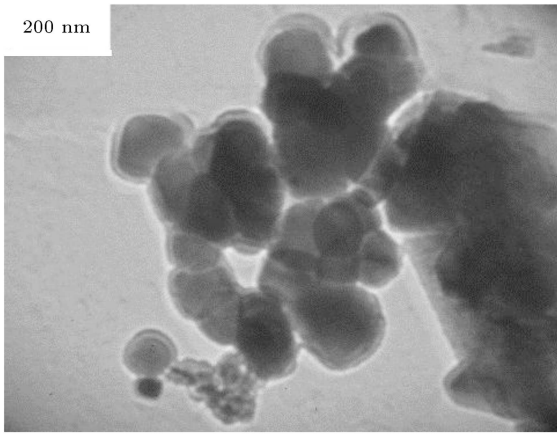


Figure 2. TEM image of 45SBG nanoparticles (particle size $\leq$ 150 nm).

Table 1. The components of the nanocomposite coatings on PLGA scaffolds.

Sample	Ha (wt.%)	Fibrin (wt.%)	BG (wt.%)
S2	50	25	25
S3	25	50	25
S4	10	80	10

PLGA scaffolds with different suspensions of Ha/ fibrin/ 45SBG. 45SBG nanoparticles were synthesized via sol-gel technique. In addition, fibrin solution was prepared with our group. A detailed procedure for 45SBG nanoparticles and fibrin solution preparation was described elsewhere [10]. For the sake of simplicity, pure PLGA scaffold (S1) and nanocomposite scaffolds were tagged as S2, S3, and S4. Details of Ha, fibrin, and 45SBG were added to the components in order to prepare nanocomposite scaffolds, as demonstrated in Table 1.

2.2. The scaffolds' degradation studies

In order to determine the in vitro biodegradability behaviour of the samples, degradation test was performed in the following. At first, PLGA and nanocomposite scaffolds were immersed (soaked) in phosphate-buffered saline solution (PBS) at pH = 7.4 and 37°C. Degradation analysis was carried out up to 1 month, taking placed every 3 days during this period's refreshment of buffer solution. In order to calculate water absorption and weight losses of the samples, three samples of each scaffold at the predetermined time periods (3, 7, 14, 21, and 28 days) were removed from PBS. Then, the wet weight of the samples was measured after carefully wiping their surfaces. At the end, the samples were dried in a vacuum oven to obtain the weight of the dried samples at 37°C for 24 h after rinsing in deionized distilled water. According to the following equations, water absorption and weight losses of the specimens were calculated [27]:

$$\text{Water absorption } (\%) = \frac{(W_o - W_a)}{W_o} \times 100, \quad (1)$$

$$\text{Weight loss } (\%) = \frac{(W_o - W_t)}{W_t} \times 100, \quad (2)$$

where W_o is the primary dry weight, W_a is the wet weight of the scaffold after removal of the PBS, and W_t is the dry weight of the sample after removal of buffer solution. Furthermore, pH variation during soaking samples in PBS was recorded.

2.3. In vitro bioactivity evaluation of the scaffolds

The in vitro bioactivity studies were conducted by immersing the pure PLGA and nanocomposite scaffolds in Simulated Body Fluid (SBF) solution. The SBF solution preparation was presented elsewhere [28]. In brief, the samples were soaked in simulated body fluid (SBF; Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 148.0, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mM) at 37°C for 3, 7, 14, 21, and 28 days. In order to reach better condition and reliable assay for apatite deposition, the solution was renewed every 3 days. Afterwards, the samples were taken out of SBF, rinsed with deionized water, and freeze-dried. Thereafter, evaluation of apatite formation was verified by Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) (Philips XL300, Holland) and X-Ray Diffraction (XRD) analyzer (Philips PW1800, Holland). In various studies, XRD is used for bioactivity assessment and apatite formation. The presentation of both XRD patterns and SEM images of the formed apatite on the scaffolds' surfaces confirmed the bioactive property of the materials [29,30].

2.4. Attachment and viability of hADMSCs on the prepared scaffolds

Human adipose-derived mesenchymal stem cells (hADMSCs) were extracted by lipectomy biopsies from subcutaneous adipose tissue of three female patients with high purity. The female patients' ages ranged from 28 to 30 years old. This protocol was performed and approved by the institutional ethical review board of Isfahan University of Medical Sciences, Iran (IRB number: 193126). In order to achieve standard stromal-cell extraction, standard protocol was applied [31,32]. After cutting adipose tissue carefully, digestion process was carried out with 0.075% collagenase type I (Sigma Chemicals, St-Louis, MO) in DMEM medium containing 10% FBS, 1% penicillin streptomycin for 60 min at 37°C. Environmental neutralization was performed with equal medium of DMEM, 10% FBS, and 1% penicillin streptomycin.

Afterwards, the medium was cast in 50 cc falcon tube, and centrifugation process was performed for 10 min with 1400 rpm. Then, floating adipocytes were scraped and added DMEM, 10% FBS to the cells deposited solution. The cells' suspension was transformed into the culture flasks and kept at 37°C, 5% CO_2 conditions. To achieve high confluence of the cells and apply it the in vitro study, suspension was passaged three times and the culture medium exchange was done every 2 days. In order to calculate cells' attachment and viability, samples were sterilized with UV radiation for 2 h and soaked in 70% ethanol overnight. A definite number of the cells were seeded onto the sterile samples. For cells' attachment and viability determination, sterile samples were transformed into six multi-wall plates and, then, seeded at a cell density of 1×10^4 cells/cm^2 in a complete culture medium. Cell culture plates were utilized as negative control. For each sample, 100-microliter cell suspension was added to each well and allowed to be attached firmly on the surfaces of the samples. Then, 3 ml of culture medium was added to every samples. Finally, after determining incubation times (3 days), a rinsing process was carried out in order to omit non-adherent cells from each well. In order to determine viable cells, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was performed. A 2 mg/ml stock solution of MTT in PBS was prepared and sterilized by Millipore filtration and kept in a dark condition before any tests. Afterwards, a certain amount of MTT solution was cast into each well, and the plates were transformed into the incubator for 3 h. Then, supernatant liquid on the surface of wells was removed. In order to dissolve the formazan crystals, dimethyl sulfoxide (DMSO) was added to each well under slight shaking. At the end, solution was transformed in 96 well plates and optical density was calculated by ELISA plate reader (Hyperion, Florida, USA) at 540 nm. The amount of absorption indicates the number of viable cells attached to each sample. Our results were recorded and reported as optical density values. Morphology of the cells incubated for 3 days was observed using SEM. Cells adhering to the substrates were washed with PBS. Subsequently, the cells were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C. The specimens, after being thoroughly washed with PBS, were dehydrated using graded ethanol changes, gold splattered in vacuum, and examined using SEM.

3. Results and discussion

3.1. In vitro biodegradation properties of the scaffolds

The in vitro degradation of pure PLGA and nanocomposite scaffolds was studied in PBS at 37°C. Degradation was carried out by measuring water absorption, weight loss, and pH variations. Figure 3 shows the water absorption in weight percentage of the pure PLGA and nanocomposite scaffolds immersed in PBS for 28 days.

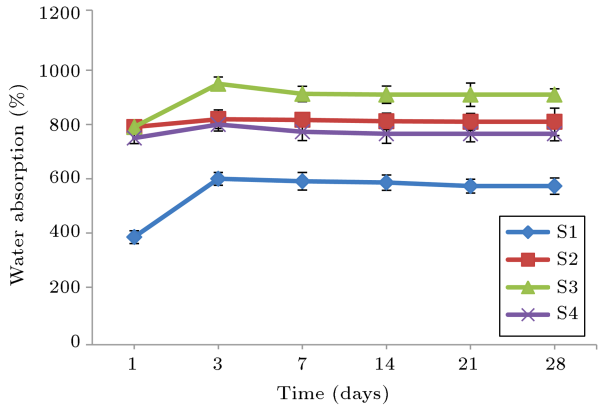


Figure 3. Water absorption vs. time for the samples soaked in PBS solution: S1 (pure PLGA), S2 (50 wt% Ha, 25 wt% Fibrin, and 25 wt% BG), S3 (25 wt% Ha, 50 wt% Fibrin, and 25 wt% BG) and S4 (10 wt% Ha, 80 wt% Fibrin, and 10 wt% BG).

It is observed that water absorption of the PLGA porous scaffold is lower than the other nanocomposite scaffolds. Results from the degradation study in PBS after 3 days of immersion for the nanocomposite scaffolds demonstrated that the water absorption slightly increased with the insertion of 45SBG nanoparticles, fibrin, and hyaluronic acid due to their hydrophilicity. Previous researches showed that nanocomposite scaffolds, containing 10 wt.% BG nanoparticles, have high level of water absorption [33]. Similarly, in this study, it was showed that S3 had the maximum water absorption. The increasing mass of pure PLGA and nanocomposite scaffolds was measured at around 600 to 900 percentage. It was concluded that the presence of fibrin, Ha, and 45SBG nanoparticles may affect the water absorption ability of the nanocomposites scaffolds due to the high tendency of 45SBG, fibrin, and Ha to absorb water [34,35]. In the nanocomposite samples, more linear and slower weight loss due to neutralizing effect of release alkaline components from the BG nanoparticles was observed which is in accordance with Orava et al. [36]. The weight loss for the pure PLGA and nanocomposite scaffolds is shown in Figure 4.

Previous study showed that higher amount of BG nanoparticles tends to form BG aggregates in the nanocomposite scaffolds and more pores' formations in their interface through which water penetrates simply [11]. This phenomenon can explain more degradation rates of S2 and S3 samples. After 28 days of incubation, S3 lost about 12% of its initial weight, which is higher than the other specimens are. For better understanding, Figure 5 illustrates

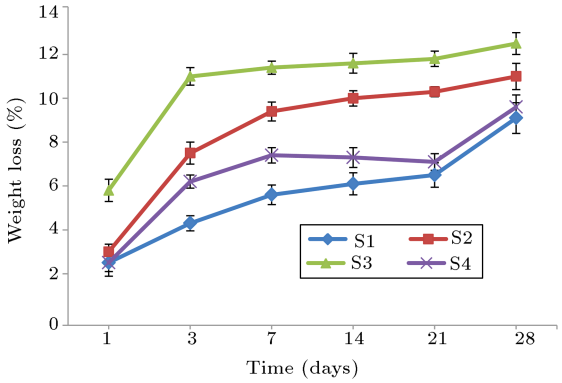


Figure 4. Weight loss vs. time for the samples soaked in PBS solution: S1 (pure PLGA), S2 (50 wt% Ha, 25 wt% Fibrin, and 25 wt% BG), S3 (25 wt% Ha, 50 wt% Fibrin, and 25 wt% BG), and S4 (10 wt% Ha, 80 wt% Fibrin, and 10 wt% BG).

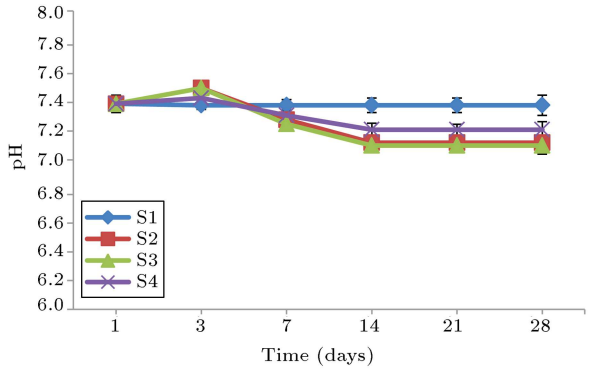


Figure 5. pH Variations vs. time for the samples soaked in PBS solution: S1 (pure PLGA), S2 (50 wt% Ha, 25 wt% Fibrin, and 25 wt% BG), S3 (25 wt% Ha, 50 wt% Fibrin, and 25 wt% BG), and S4 (10 wt% Ha, 80 wt% Fibrin, and 10 wt% BG).

the degradation process, pH variation of the PBS environment as a function of immersion time.

The pH values of the nanocomposite scaffolds with different amounts of 45SBG nanoparticles (10-25 wt.%) displayed a slight increase of pH in the early days of incubation time and, then, a decrease around 7 to 28 days. Literatures have reported that overloading contents of BG (up to 30 wt.%) could accelerate the degradation rate of nanocomposite scaffolds [33,11]. Furthermore, in this work, it was demonstrated that the nanocomposite scaffolds with 25 wt.% 45SBG nanoparticles could obtain hydrophilic surface and large specific surface area and release more acidic products into the medium, which might result in the enhancement of degradation rate of the PLGA matrix. Complete regeneration of cartilage tissue is different depending on several factors such as defects size, shape, location and depth of the lesion. According to a scientific report, it takes approximately 12 weeks for cartilage regeneration. The prepared nanocomposite

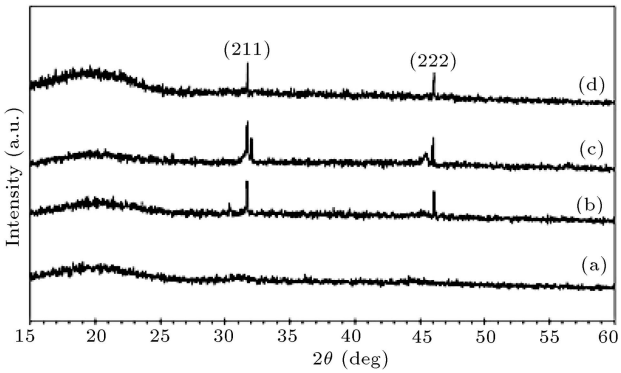


Figure 6. The XRD pattern of the prepared scaffolds after immersion in SBF solution for 7 days: (a) S1 (pure PLGA), (b) S2 (50 wt% Ha, 25 wt% Fibrin, and 25 wt% BG), (c) S3 (25 wt% Ha, 50 wt% Fibrin, and 25 wt% BG), and (d) S4 (10 wt% Ha, 80 wt% Fibrin, and 10 wt% BG).

scaffolds illustrated the entire degradation for 12 weeks. Therefore, they could be suitable for this purpose [37].

3.2. In vitro bioactivity evaluation

To determine the bioactivity of the prepared pure PLGA and nanocomposite scaffolds, the samples were soaked in SBF solution. Apatite formation was studied using XRD analysis (Figure 6).

According to the XRD analysis using X-ray diffractometer, semi-sharp peaks of apatite crystals were observed. After 7 days of incubation time, peaks (211) and (222) at 32° and 46° belong to the formed apatite phase which were in accordance with the data of Nazemi et al. [38]. With this characteristic peaks, the formation of the apatite on the surface of the pure PLGA and the nanocomposite scaffolds was confirmed. Researchers have shown that the soluble products from BGs degradation can enhance hydroxyapatite formation on the surface of scaffolds and provide suitable environment for neocartilage formation in vitro studies [39]. Furthermore, the apatite formation was confirmed with the SEM images. Figure 7 shows the SEM images and EDS analysis of the prepared scaffolds' surfaces after immersing in SBF solution.

The images showed that, after 7 days, hydroxyapatite particles were formed on the surface of the samples. EDS confirmed hydroxyapatite sedimentation, and Ca/P ratio was about 1.4. These results are in accordance with the results obtained from XRD analysis of different samples. It can be observed that there were no apatite crystals on the surfaces of the pure PLGA samples after 7 days of incubations in SBF, and no apatite peaks were found in XRD pattern of such samples. In fact, AC and subchondral bone are the complex structures. From this point of view, as those researchers mentioned, the presence of sufficient amount of hydroxyapatite on the surface of regenerative construct could affect cell attachment and mimic

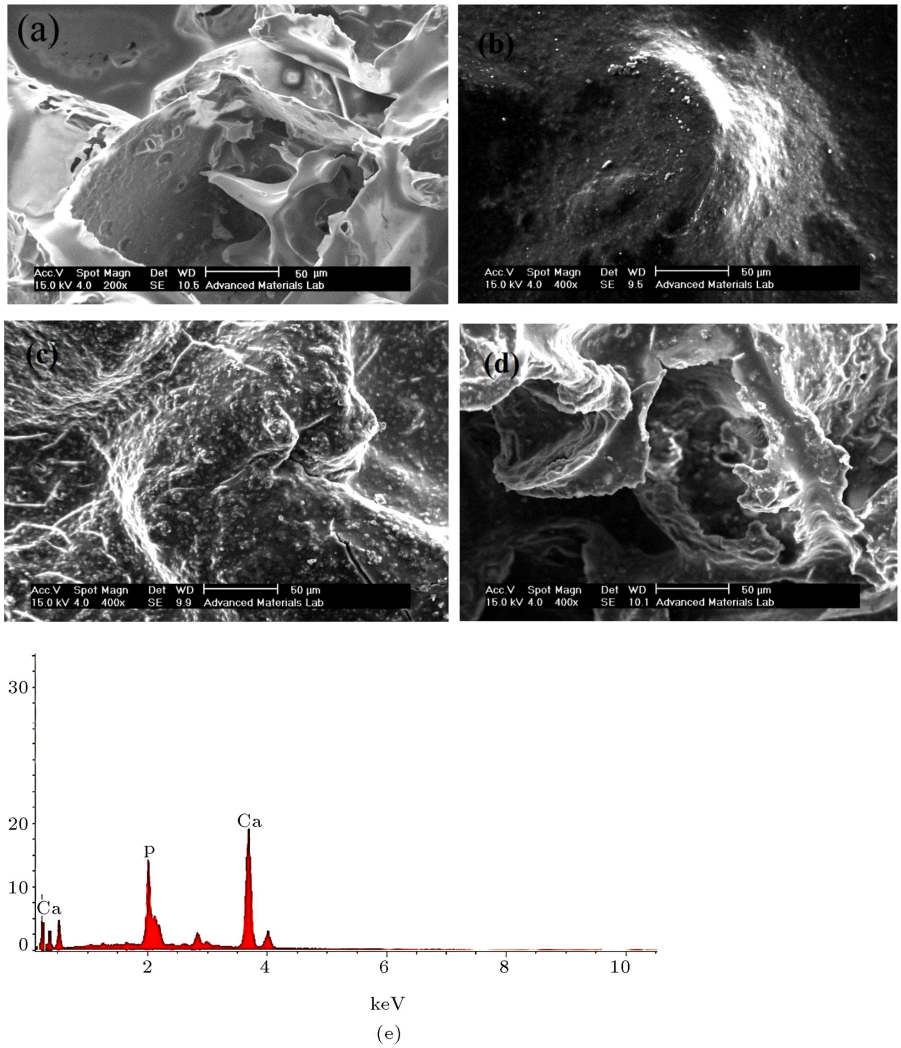


Figure 7. SEM images of the prepared scaffolds surface after soaking in SBF solution for 7 days: (a) S1 (pure PLGA), (b) S2 (50 wt% Ha, 25 wt% Fibrin, and 25 wt% BG), (c) S3 (25 wt% Ha, 50 wt% Fibrin, and 25 wt% BG), (d) S4 (10 wt% Ha, 80 wt% Fibrin, and 10 wt% BG) and Scaffold structures and porosities, (e) EDS analysis of the hydroxyapatite formed on the nanocomposite scaffold surfaces (S3).

native calcified cartilage and may be considered as a prominent way to rehabilitate damaged cartilage [40].

3.3. Viability and proliferation of the hADMSCs on the prepared scaffolds

Figure 8 shows MTT assay results of the samples after three different incubation periods (24, 48, and 72 h). Significant statistical differences were observed after 24 h of incubation between the pure PLGA and nanocomposite scaffolds with control ($P < 0.05$). Furthermore, the same results were obtained for the pure PLGA and nanocomposite scaffolds at 48 h and 72 h. Greater proliferation and viability of the hADMSCs were observed on the nanocomposite scaffolds than on the pure PLGA scaffold and the control.

Figure 9 shows morphology of the stem cells attached to the scaffold surface after 3 days of incubation. The scaffold supported the attachment of hADMSCs.

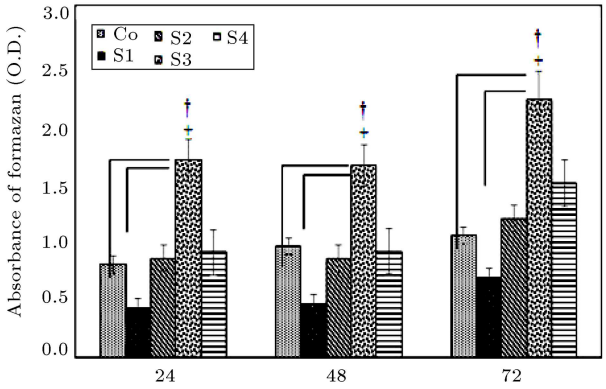


Figure 8. MTT assay results of hADMSCs seeded on the pure PLGA and nanocomposite scaffolds. The number of viable cells is proportional to the absorbance values. Significant statistical differences between the nanocomposite scaffolds and S1 (pure PLGA) $+p < 0.05$, and negative control $p\dagger < 0.05$ were determined.

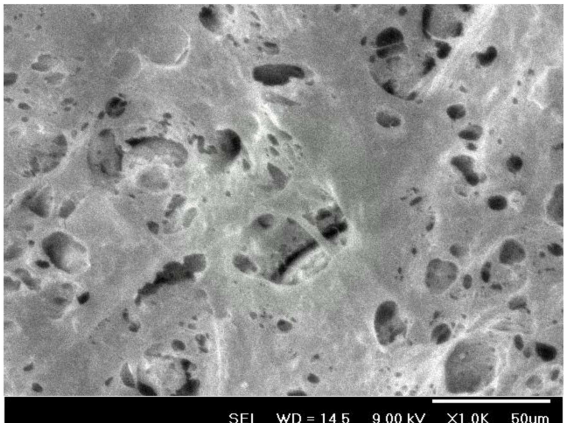


Figure 9. SEM images of the morphology of the stem cells attached to the surface of scaffold S3 (25 wt% Ha, 50 wt% Fibrin, and 25 wt% BG) after 3 days of incubation. The scaffold supported the attachment of hADMSCs.

Attachment of hADMSCs onto the nanocomposite scaffolds was approved. It was reported that the existence of BG particles could enhance cell adherence and spreading. The nanocomposites with BG nanoparticles were hydrophilic, which could make a surface more wettable than pure PLLA did [11]. The synergetic effects of the 45S BG nanoparticles, fibrin glue, and hyaluronic acid on the surface of the scaffolds could enhance early cell capture, proliferation, and differentiation.

In fact, compared to the pure PLGA scaffold and the control, more viability and proliferation of the cultured cells were observed in the nanocomposite scaffolds with increasing the amount of Ha, fibrin, and 45SBG nanoparticles. As a consequence, the presence of 45SBG nanoparticle in the nancomposite construct and their ionic products released from them in medium could induce cell proliferation. These results are in compliance with several publications [11,12]. Furthermore, from the other point of view, the presence of Ha and fibrin could promote cell attachment, viability, and proliferation. The presence of 45SBG nanoparticles in the nanocomposite scaffolds could enhance more cell attachment on the surface of the samples. Therefore, a suitable environment is created for cultivated cells. This is in agreement with previous studies' data where they approved the role of Ha and fibrin in providing the good condition for cell viability [40,41].

The current understanding is mainly reported to involve the interaction of stem cells with the nanocomposite scaffolds by motivating various signaling pathways. Substrate surface and scaffold bulk properties are also reported to affect not only short-term stem cell attachment, spreading and proliferation, but also longer-term lineage differentiation, functionalization, and viability [42].

Previous studies showed that the incorporation of BG into a polymer matrix effectively improved both the hydrophilicity of the nanocomposites and the amount of attached cells. In an animal study, they could form a thicker cartilage-like tissue with better biomechanical properties and a higher cartilage matrix amount than the scaffolds made by pure polymer.

4. Conclusion

Due to unsatisfactory results obtained from cartilage treatment with conventional techniques, innovative solutions, such as combination of naturally and synthetically polymers with the presence of a bioceramic phase to make a suitable scaffold, have gained a huge attention for cartilage tissue engineering. In this study, PLGA/Ha/fibrin/45SBG (S3) nanocomposite scaffold with the composition of 25 wt% Ha, 50 wt% fibrin, and 25 wt% 45SBG showed good bioactivity, suitable biodegradability, and excellent cell response, including high viability and attachment. Therefore, a promising construct can be proposed for cartilage tissue engineering. However, in the next step, more tests should be performed to confirm the good potential of such a scaffold for chondrogensis.

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