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Preparation of Diatom-Doped Bio-Nanocomposite Materials for Bone Tissue Scaffolds

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Naturally sourced materials have an important place in bone tissue engineering due to their biocompatibility and biodegradability. Non-diatom, diatom-doped chitosan/hydroxyapatite (CS/HAp) and collagen/chitosan/hydroxyapatite (Col/CS/HAp) as three-dimensional tissue scaffolds were produced by freeze drying technique. It was determined by SEM analysis that CS/HAp, CS/HAp/Di, Col/CS/HAp, Col/CS/HAp/Di scaffolds have 160 µm, 130 µm, 390 µm and 340 µm pores, respectively. The diatoms in the structure have approximately 9-16 µm in length, 8-20 µm in diameter and nanopore sizes of 260-330 nm. Cell culture studies were performed using the 3T3 cell line to study the non-toxic nature of biocomposite scaffolds that support cell attachment and proliferation. The cells in the scaffolds without diatom proliferate in a reticulated manner, whereas in the scaffolds containing diatom the cells were wrapped around the scaffold like a cover. The suggested scaffolds have the potential to meet the basic requirements in biocompatibility, cytocompatibility and interconnected pore structure.

Keywords: *Bio-nanocomposite, Bone-tissue, Diatom, Scaffold, Tissue engineering scaffold.*

1. Introduction

Large numbers of people suffer from bone defects caused by accident, injury, trauma, tumors, or bone-related diseases[1]. Bone is a living and porous tissue that protects the body's organs and soft tissues. It functions as blood production and mineral reserves[2]. As is known, biologically produced bone forms have self-healing properties. In addition, large bone defects do not heal spontaneously and require surgical intervention for treatment[3]. Clinically used bone grafts can be divided into two main types, biological and synthetic, according to their origin.

Disadvantages of biological grafts may include immune rejection and biocompatibility issues[4]. In order to solve these problems, suitable synthetic materials are being researched more and more every day. Bone tissue engineering, which works towards the production of synthetic grafts that includes intradisciplinary sciences, plays an important role in achieving this goal[5,6].

Bone tissue engineering aims to simulate the tissues in biological systems in the best way by using bone tissue scaffolds, cells and growth factors alone or together to repair bone damages or reconstruct damaged tissue[7]. Polymers and their derivatives have been investigated in many areas since 1990, because of their environment-friendly nature and practical applications in biomedical, pharmaceutical, and cosmetic fields from micro to nanoscale engineering[8,9]. In doing so, it uses biocompatible polymers that can mimic the extracellular matrix and tries to regenerate or repair damaged or dysfunctional tissue skeletons in the human body[10]. The most important feature of tissue scaffolds to be used in bone tissue engineering is that they are osteoinductive and/or osteoconductive. Osteoinductive tissue skeletons allow osteoprogenitor cells to attach to the scaffold, migrate to the scaffold, differentiate and ultimately form new bone. Osteoconductive tissue scaffolds enable the formation of the 3-dimensional structure of the bone by supporting the formation of the capillary structure of the bone and the proliferation of bone cells by directing the cells from the main tissue to the material[11-13]. Apart from these features, the tissue scaffolds to be used must be biocompatible, porous and biodegradable and at the same time have sufficient mechanical strength since they will be exposed to mechanical stress when implanted in the body.

Hydrogel, a natural 3D scaffold, can be prepared from synthetic or natural polymers. Compared to synthetic hydrogels, natural hydrogels tend to have greater inherent biocompatibility and desirable biodegradability[14-16]. Hydrogels obtained from various materials emerge as an approach in biomedical in the field of tissue engineering. Recent research shows that it focuses on hydrogels carried out biological materials. These hydrogels can be prepared from protein structured polymers such as collagen, gelatin, and/or polysaccharide polymers such as chitosan, alginate[17,18]. In addition, bio-ceramic hydroxyapatite and diatom with a bioactive structure in the composite hydrogel structure prepared to form the tissue scaffold can also be used[19].

Diatoms are unicellular eukaryotic organisms that live in aqueous environments and are the largest source of biosilica formation. Diatoms of a wide variety of shapes form an amorphous silica shell with symmetrically dispersed nano-micropores with high mechanical stability[20,21]. Diatom is a cheap and unlimited source of biogenic silica[22,23]. Thanks to its unique porosity and morphology, it has been proposed for use in drug delivery systems, bio-fixing agents, molecular catalysis and photonics applications[24-26]. As silicon plays an important role in bone formation, regeneration and mineralization, we think diatom, an inexpensive source of

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silica, will be a promising natural resource in bone tissue engineering. As a result of the research we have done, we see that our opinion is supported by encountering a very limited number of studies aimed at this purpose in recent years[20-22]. In addition to inorganic materials such as hydroxyapatite, zirconia, glass ceramic, tri-calcium phosphate, the use of amorphous silica particles has been proposed to support mineralization in bone regeneration studies[22,27,28]. In addition, silica has been used successfully with hydroxyapatite to increase osteo conductivity for bone regeneration[29,30].

Hydroxyapatite has similar components and structure to natural bone. It also has good biodegradability, biocompatibility and osteoconductivity. It also has the function of absorbing and accumulating calcium ions in body fluids and can support bone regeneration in polymer-based composites[31,32]. There is evidence that HAp plays a role in biological processes such as angiogenesis, wound healing, ECM (Extra Cellular Matrix) organization, and inflammation[33]. HAp derivatives are successfully used as scaffolding materials to treat vascular diseases due to their properties of bone and skin tissue regeneration, chondrocyte growth, biocompatibility and anti-inflammation[34]. However, its use alone in tissue engineering applications is limited due to its non-biodegradable, poor mechanical properties, and processing difficulties[35]. Therefore, hydroxyapatite is used by being included in composite polymer hydrogels.

Chitosan is the second most abundant bio-polysaccharide in the world, created by the deacetylation of chitin produced from shellfish, insects and fungi[36]. Chitosan is currently a material of great interest in tissue engineering[37]. The mechanical and physical properties of the hydrogel formed are directly related to the deacetylation degree and molecular weight of chitosan. Chitosan used in composite hydrogels has low cost, antibacterial, biodegradable, biocompatible and bioactive, easy to sterilize preparties. All these features can be controlled by changing the deacetylation level[38]. Their disadvantages are that they are easily affected by parameters such as pH and temperature. It also shows poor mechanical properties[39]. Therefore, it must be compounded with other materials such as hydroxyapatite, calcium phosphate, gelatin and alginate while forming a hydrogel[40].

Collagen is the most abundant ECM protein and provides an appropriate environment for cell adhesion and signaling molecules[41,42]. Until now, collagen has been used in the repair and renewal of many tissues such as bones, skin and heart[43]. Biologically, collagen has positive properties such as low inflammatory response and low antigenicity, biodegradability and biocompatibility[44]. Collagen and collagen-based materials play an important role in maintaining the structural integrity and biological function of tissues. Therefore, it is widely used in tissue regeneration and tissue engineering studies[45]. Unlike collagen in natural tissues, mechanical strength is insufficient in collagen-based biomaterials for the absence of covalent crosslinking. Because the crosslinking is performed by various methods to increase the mechanical performance of the tissue[46]. Besides these, many studies have been done using various manufacturing techniques, including different synthetic materials and/or combinations with biomolecules[47].

In recent years, biopolymers have been used together with nanomaterials on bone tissue engineering studies. The freeze drying technique has been the subject of many research in terms of its ease and successful results. The scaffolds based on the cellulose-graft-polyacrylamide/nHA semi-IPN nanocomposite can bind to living bone through the formation of apatite layers on its surface can be used in bone tissue engineering[48]. Chitosan/Alginate/Diatom scaffolds have been fabricated an alternative potential in the field of tissue engineering because of its high porous and non-toxic properties[49]. Nanoclay particles were incorporated into polyvinyl alcohol–chitosan to improve the mechanical properties and bioactivity for bone tissue replacement applications[50]. Novel biocompatible nanocomposite scaffolds have been prepared by freeze drying method using TiO_2 doped in grafted chitosan/hydroxyapatite for bone tissue engineering applications[51]. Alginate and hyaluronic acid hydrogel polymers reinforcing with titanium oxide nanoparticles has been developed for orthopedic field. This nanocomposite was prepared using freeze drying technique[52]. Hydroxyapatite and polymethylmethacrylate was fabricated to obtain porous polymeric-ceramic material[53].

Designing biomaterials for bone tissue engineering applications is still a challenge regarding the natural complex structure of hard tissues. In this study, in addition to chitosan/hydroxyapatite and gelatin/chitosan/hydroxyapatite hydrogels, diatom doped forms of these hydrogels were prepared. The liquid part of these four different bio-composite hydrogels, which were created to take advantage of the best properties of each material, was completely removed by freeze drying, and as a result, porous, 3-dimensional scaffolds were formed. Scaffolds produced in this combination for the first time were examined by SEM, FT-IR and cell culture studies.

2. Experimental Section

2.1. Materials

Chitosan (medium molecular weight) was purchased from Sigma-Aldrich (product of Iceland). Hydroxyapatite (nano-powder), Collagen Type-I (from calfskin) and diatomaceous earth (suitable for most filtrations) were purchased from Sigma-Aldrich (USA). Acetic Acid (Glacial, %100 Anhydrous), a solvent for chitosan and collagen, was obtained from ISOLAB chemicals (Wertheim, Germany). Glutaraldehyde used in crosslinking hydrogels was purchased from Merck (USA). DMEM (Dulbecco's Modified Eagle Medium), which was used as a medium for cell culture studies, was purchased from Sigma–Aldrich (USA). 3T3 cell line was purchased from the European Validated Cell Culture Collection (ECACC). All chemicals used in this study were at the analytical level.

2.2. Preparation of scaffolds

Chitosan and Collagen solution; Chitosan (3%, w/v) was dissolved in 1% acetic acid solution, which was stirred for 24 hours at 50 °C. Collagen (2.5%, w/v) was prepared by stirring in 1% acetic acid for 12 hours at 40 °C. With the preparation of chitosan and collagen solutions, biocomposite hydrogels with four different contents were prepared. The term BTS is encoded as the abbreviation of Bone Tissue Scaffold. In our study, four different mixtures have been

called BTS-1 (CS/HAp), BTS-2 (CS/HAp/Di), BTS-3 (Col/CS/HAp) and BTS-4 (Col/CS/HAp/Di). The preparation of these four mixtures is given below, respectively.

BTS-1: 2g of hydroxyapatite was added to 20 ml of chitosan solution and mixed until homogeneous.

BTS-2: 0.1 g of diatom was added to 20 ml of BTS-1 solution and mixed until homogeneous.

BTS-3: 10 ml of chitosan and 10 ml of collagen solution was mixed and 2 g of hydroxyapatite was added to this solution and mixed until homogeneous.

BTS-4: 0.1 g of diatom was added to 20 ml of BTS-3 solution and mixed until homogeneous.

In summary, Table 1 provides information on the materials contained in each composite scaffold.

These four hydrogels were kept overnight at -20 °C after crosslinking with glutaraldehyde (2.5%, v/v). It was then lyophilized (freeze dried) for 48 hours. In Figure 1, the visual of the scaffolds formed after the freeze drying (lyophilization) (Labconco FreeZone -105 °C, USA) process is given.

Table 1. Bio-composite scaffolds and their ingredients.

	CS	HAp	Col	Di
BTS-1	✓	✓		
BTS-2	✓	✓		✓
BTS-3	✓	✓	✓	
BTS-4	✓	✓	✓	✓

2.3. Characterization studies

Scanning electron microscopy (SEM) (Zeiss Supra 40VP, Germany) was used to view the morphology of composite scaffolds before and after cell culture[54]. The chemical bonds and functional groups of biocomposite tissue scaffolds were examined using Fourier Transform Infrared Spectroscopy (FT-IR) (Thermo Scientific Nicolet iS50, Germany) at a resolution of 0.5 cm^{-1} and a frequency of 400-4000 cm^{-1}.

2.4. Cell culture studies

2.4.1. Cell line preparation and maintenance

Studies of cell culture were performed with the 3T3 mouse embryonic fibroblastic cell line (ECACC, UK). Cells were cultured in Petri dishes using Dulbecco's modified Eagle's medium (DMEM; Sigma, Germany) containing 10% FBS and 1% penicillin-streptomycin. Cells were subcultured every two days by keeping them in an incubator (EC-160, Nüve, Turkey) with 37°C, 95% humidity and 5% CO_2 environment prior to seeding.

2.4.2. Cell seeding into tissue scaffolds

Cell culture was carried out in sterile well culture dishes. Before cell seeding, the bottom of the culture dishes was covered with parafilm and washed with alcohol. Thus, the bottom of the cell culture dish was made hydrophobic, preventing the cells from migrating from the tissue scaffold to the surface of the culture dish. On the other hand, biocomposite bone tissue scaffolds were washed with 70%

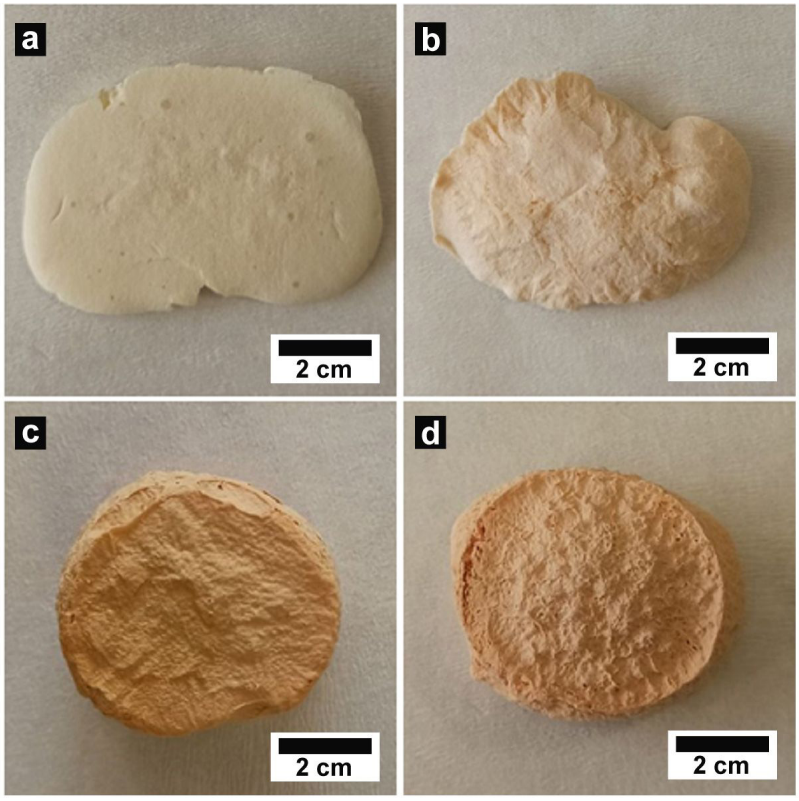

Figure 1. Bone tissue scaffolds formed after freeze drying: (a) BTS-1, (b) BTS-2, (c) BTS-3 and (d) BTS-4.

alcohol and left to dry. Then scaffolds and culture dishes were sterilized under UV light for 45 minutes. Before cell seeding, scaffolds were placed in culture dishes and kept in DMEM for 24 hours to interact with serum proteins. At the end of 24 hours, $1x10^4$ cells were seeded in each medium containing a scaffold. During 8 days[55] of cell culture, the culture medium was renewed every 2-3 days.

2.4.3. Morphological analysis

The culture medium on the tissue scaffolds was removed and the scaffolds were washed twice with PBS (Biowest, France). The cells were fixed by soaking the tissue scaffolds in 2.5% (v/v) glutaraldehyde solution for 30 minutes. The scaffolds were kept in 30%, 50%, 70%, 90% and 100% (v/v) ethanol solutions for 2 minutes, respectively, and dehydration was performed[56]. It was then kept in hexamethyldisilazane (HMDS; BRB, Netherland) for 5 minutes and allowed to dry at room temperature. Scaffolds were made conductive by coating with gold-palladium for 400 seconds for SEM analysis.

2.4.4. Cytotoxicity studies

Cytotoxicity tests were performed to determine whether bone scaffolds have cytotoxic potential. Mouse embryonic fibroblast cells (3T3) were used for this purpose. This cell line was used for many purposes including biomaterial science[57]. Briefly, $2x10^3$ cells were seeded in each wells of 96-well plate in DMEM containing 10% FBS and 1% penicillin/streptomycin mixture with the humidified atmosphere (95% air with 5% CO2). The medium was removed after 24 h and medium containing different amounts of BTS samples extracted by the methods of Lin et al. (2013)[58] with slight modifications. After 24 hours of treatment, cell viability was determined by MTT method as described by Konus et al. (2020)[59].

2.4.5. Statistical analysis

All experiments were run in triplicates. Statistical analyses were performed using Student's ttest for multiple comparisons (Minitab Software). Data are expressed as that mean value (±SD*P<0.05) is considered significant

3. Results and Discussion

3.1. SEM analysis

As seen in Figure 2, SEM images of non-diatom BTS-1 and BTS-3 scaffolds are given at different magnifications. Here, it is seen that BTS-1 and BTS-3 scaffolds have approximately 160 µm and 390 µm macroporosities, respectively. It has been observed through SEM that the BTS-3 scaffold, unlike BTS-1, has larger pores due to the collagen it contains.

In Figure 3, SEM images of diatom containing BTS-2 and BTS-4 scaffolds are given at different magnifications. Unlike BTS-1 and BTS-3 scaffolds that do not contain diatom, it has been observed that these scaffolds have macro and micro pores as well as nanopores originating from diatom. It has been observed that BTS-2 and BTS-4 scaffolds have pores from macro to nano at different magnifications. Here, it is seen that BTS-2 and BTS-4 scaffolds have approximately

130 µm and 340 µm macroporosities, respectively. It has been shown through SEM that the BTS-4 scaffold, unlike BTS-2, has larger pores due to the collagen it contains. It has been seen that the same type of diatoms found in these scaffolds are approximately 9-16 µm in length and 8-20 µm in diameter. It has also been observed that these diatoms have nanopore sizes of 260330 nm at regular intervals.

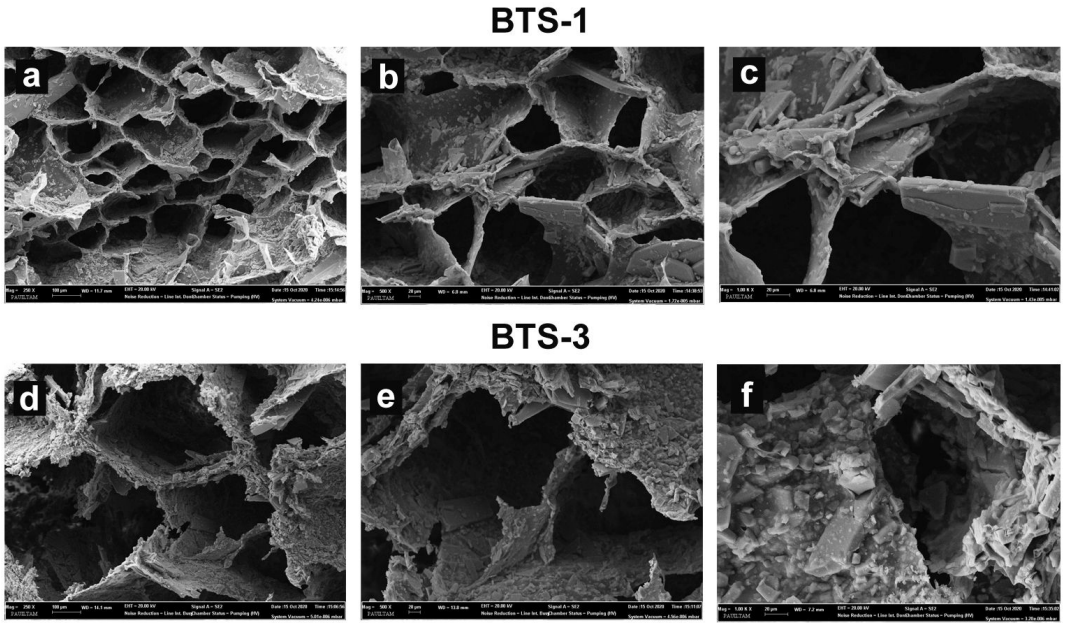

Figure 2. Morphological images of non-diatom BTS-1 scaffold (a) 250x, (b) 500x, (c) 1000x and BTS-3 scaffold (d) 250x, (e) 500x, (f) 1000x.

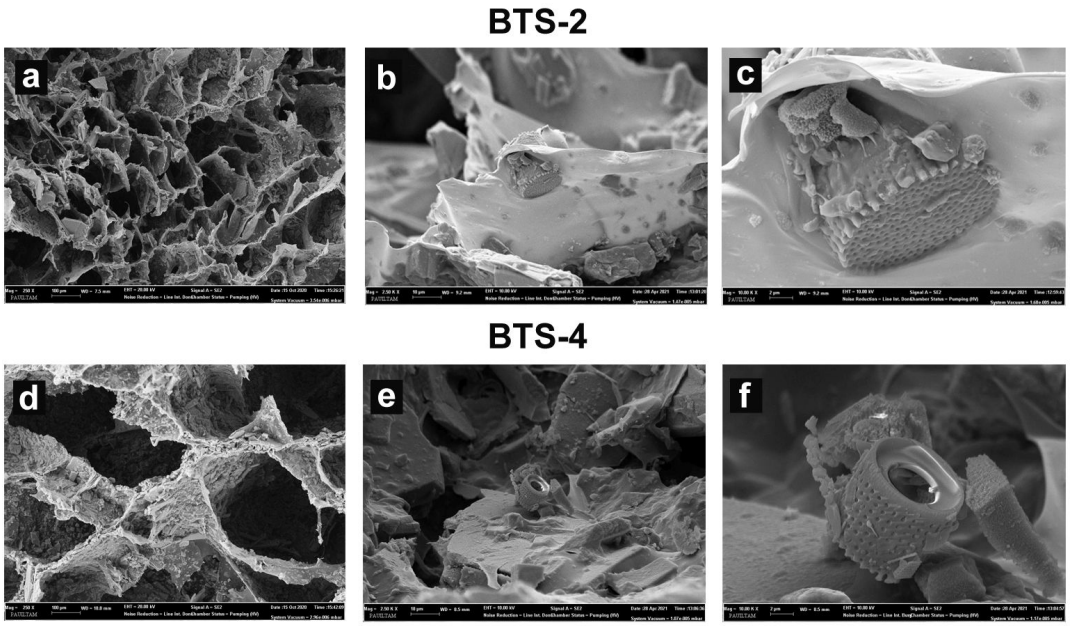

Figure 3. Morphological images of diatom BTS-2 scaffold (a) 250x, (b) 2500x, (c) 10000x and BTS-4 scaffold (d) 250x, (e) 2500x, (f) 10000x.

3.2. FT-IR analysis

FT-IR result graph of bone tissue scaffolds is given in Figure 4. The FT-IR data of the structures forming the scaffolds are given separately. It was determined that the OH groups of HAp in the

BTS-1, BTS-2, BTS-3 and BTS-4 scaffolds were located in the asymmetric stretch band at 3288, 3290, 3291, 3284 cm^{-1} levels, and in the asymmetric bending band at 1647, 1652, 1652, 1647 cm^{-1} levels, respectively. It was determined that the PO_4^{-3} groups of HAp were located in the asymmetric stretch band at the levels of 559, 558, 558,559 cm^{-1} and in the asymmetric bending band at the levels of 1016, 1012, 1015, 1016 cm^{-1}, respectively[60-62].

The 892-1152 cm^{-1} characteristic peaks of chitosan are located in the C-O-C asymmetric stretch band. In addition, it was observed that C=O stretching (amide I) at the 1633 cm^{-1} level and NH bending (amide II) band at the 1537 cm^{-1} level. The collagen specific 1630, 3324 cm^{-1} levels were located in the N-H stretching (amide I) band, 1543 cm^{-1} level was located in the C-N stretching and N-H bending (amide II) band. The peak in the range of 1076-1100 cm-1 of the diatom shows the vibrations in the asymmetric Si-O Si bonds, and the peak in the range of 750850 cm^{-1} shows the vibrations in the symmetrical Si-O-Si bonds. The peak at 600 cm^{-1} levels is due to the crystal structure of the diatom[60-64].

FT-IR results show that the prepared compounds are rich with in functional groups such as carboxylic, amino and amide groups. Characteristic bands of both hydroxyapatite and chitosan compounds were present in the material. Depending on the chemical bonds between chitosan and calcium/phosphate ions, phosphate ions and calcium can be homogeneously retained in the polymerized precursor on a molecular scale.

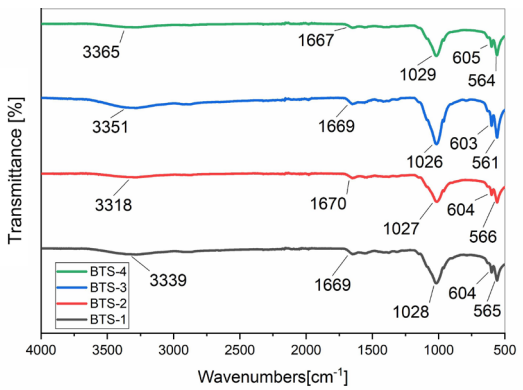

Figure 4. FT-IR graph of biocomposite scaffolds: (a) BTS-1, (b) BTS-2, (c) BTS-3 and (d) BTS-4.

3.3. Results of cell culture studies

In this study, cell culture studies were carried out under stagnant conditions for 8 days with the 3T3 cell line using the tissue scaffolds synthesized. Viability, attachment and morphology of 3T3 cells on the prepared scaffolds were investigated by the analyzes made during the culture and the effect of diatom-doped scaffolds on differentiation was evaluated by comparing them with other scaffolds.

The morphology of day 8 of 3T3 cells cultured on the produced tissue scaffolds is given in Figure 5. It is clear that cells attach to the scaffold, spread out and migrate into intrinsically linked pores. It was observed that the cells in the scaffolds without diatom proliferate in a reticulated manner, whereas in the scaffolds containing diatom the cells were wrapped around the scaffold like a cover. It is understood that diatom supports more cell proliferation thanks to its bioactive property.

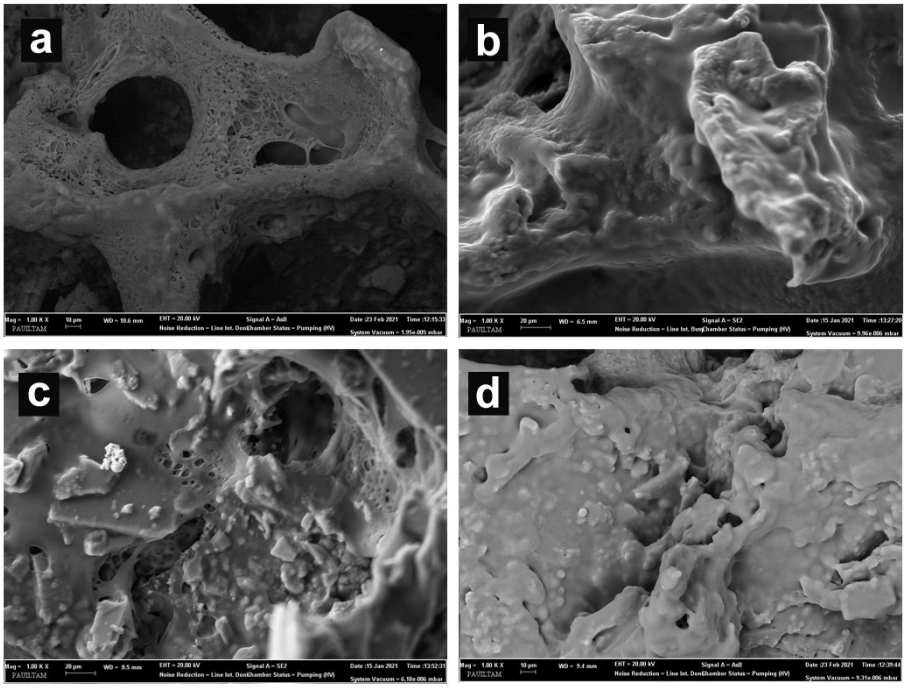

Figure 5. SEM images of diatom-free BTS-1 (a) and BTS-3 (c) scaffolds and diatom-doped BTS-2 (b) and BTS-4 (d) scaffolds with 3T3 cells after 8 days of cell culture.

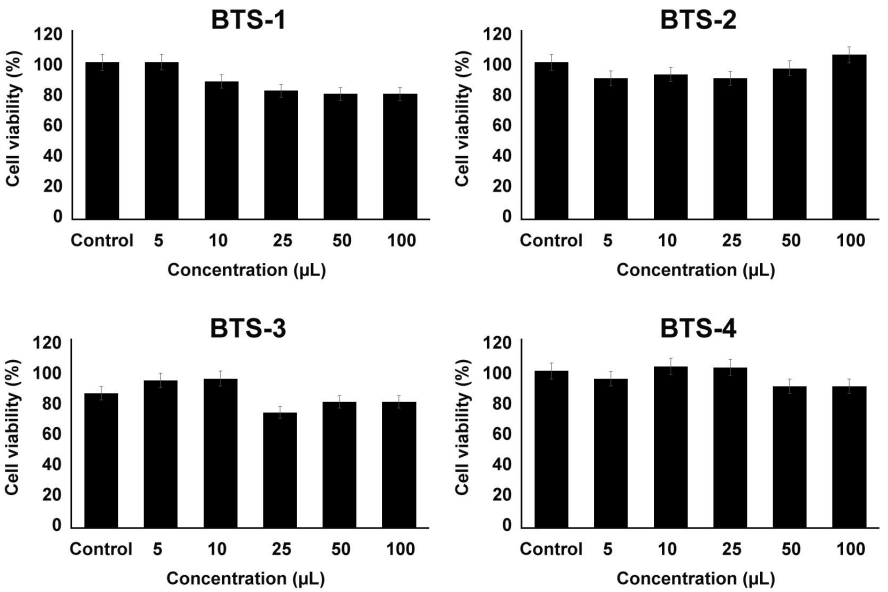

Figure 6. Effect of scaffolds at different concentrations on 3T3 cell viability.

3.4. Results of cytotoxicity studies

As a result of the studies obtained, effective doses were found for the 3T3 cell line with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, as seen in Figure 6. The results are the mean values of the triplicate measurement of two different cytotoxicity assays.

It was determined that BTS scaffolds used at different doses did not cause suspicious toxic effects on cells. The viability of cells was decreased slightly at higher doses but they were not found statistically significant (Figure 6).

4. Conclusions

This work aimed to investigate the properties of a biocomposite based on blends of biopolymers and hydroxyapatite with the addition of diatom to develop potential scaffold material. The use of biocomposite structures, such as collagen,

chitosan, hydroxyapatite and diatom for scaffold preparation by freeze drying is beneficial because it can increase the biocompatibility of the material, physical and chemical properties. Looking at the morphological observation by SEM of the scaffolds, it was observed that the macropores of the scaffolds containing diatoms were slightly smaller than the scaffolds without diatoms and diatom-specific nanopores were observed. It was determined that the macropores of the collagen-containing scaffolds were larger. These pores are critical in cell attachment, differentiation and ECM formation. In addition, diatom-specific extra nanopores will further support these effects. In addition, the FTIR analysis that the asymmetric tension and bending bands of the structural bonds in the tissue scaffolds were inconsistent peaks.

As a result of cell culture studies, while cells proliferated in a reticulate manner in diatom-free scaffolds, cells in diatom-containing scaffolds completely covered the scaffolds like a cover. It is thought that the bioactive feature of the diatom promotes cell adhesion and proliferation more. Ultimately, it was determined that the scaffolds were highly biocompatible and have ideal pores as the cells proliferated by adhering to the scaffolds.

As a result of the cytotoxicity studies, it was concluded that BTS-1, BTS-2, BTS-3 and BTS-4 scaffolds did not cause toxic effects on 3T3 cells at any of the applied dose levels. With these results, the scaffolds make a positive contribution to the literature with the materials they contain. Diatom can be used as a source of bioactive silica with its unique nanopores in order to improve the osteoinductive properties of tissue scaffolds in bone tissue engineering studies.

The developed biocomposite scaffolds have promising potential for bone tissue engineering.

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