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RESEARCH ARTICLE

# Development and investigation of novel alginate-hyaluronic acid bone fillers using freeze drying technique for orthopedic field

***Sepehr Jamnezhad[1], Azadeh Asefnejad[1], Mehdi Motififard[2,*], Hassan Yazdekhasti[3], Amin Kolooshani[4], Saeed Saber-Samandari[5], Amirsalar Khandan[5,*]***

*[1] Biomedical Engineering Department, Islamic Azad University Science and Research Branch, Tehran, Iran*

*[2] Department of Orthopedic Surgery, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

*[3] Student Research Committee, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

*[4] Mechanical Engineering Department, Khomeinishahr Branch, Islamic Azad University, Khomeinishahr, Iran*

*[5] New Technologies Research Center, Amirkabir University of Technology, Tehran, Iran*

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**ABSTRACT**

Cartilage tissue has a low cell population with a dense extracellular matrix (ECM) and is also devoid of neurons as well as blood and lymph vessels. Bone tissue is able to heal itself but in cases of serious damage and auxiliary treatment methods are necessary. Nevertheless, they have their own restrictions and downsides. Tissue engineering is working towards overcoming these challenges using 3D printing and freeze-drying technique. This research project aims to develop and study the properties of a freeze-dried antibacterial tissue based on alginate, hyaluronic acid and titanium dioxide nanoparticles using freeze drying technique. The mechanical evaluations showed that the addition of titanium dioxide improved tensile strength, hardness and wettability of the antibacterial nanocomposite scaffold. The biological assessments of the sample were evaluated in the simulated body fluid to stimulate the hard tissue reaction with biological environment. The samples were characterized using X-ray diffraction (XRD) and scanning electron microscopy (SEM) analysis. The obtained results indicated that addition of titanium oxide nanoparticle improved the hyaluronic acid polymer for bone filler using for orthopedic applications. The XRD analysis did not detect the formation of any new unwanted chemicals in the composite samples. The microscopic assessments confirmed the formation of nanocomposite scaffold containing titanium dioxide nanoparticles, with a porosity percentage between 77% and 82%. The phase analysis confirmed the triploid amorphous structure showing a significant resemblance to natural human bone tissue, thus supporting the idea of using this biomaterial as a multilayer bone filler.

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## INTRODUCTION

Human bones play an important role both in keeping the body's structural stability and the ability to move. Bone tissue heals itself when it is damaged, but auxiliary treatment methods improve the healing process a lot and they might be essential for treating severe wounds [1-2]. One of the examples of such auxiliary treatment is bone tissue transplants, however, there are complications such as a scarcity of graft and damaging the source tissue. Furthermore, there is a long history of using autologous and allogeneic transplants to cure structural defects. Even though autologous transplants have an advantage in biocompatibility, there's a need for additional surgery to extract a

* Corresponding Author Email: *motififardorthoo@med.mui.ac.ir*
*amir_salar_khandan@yahoo.com*

graft from the donor's tissue in orthopedic domain [3-4]. Also, in allogeneic transplants, there is a risk of infection, graft rejection and impact on the recipient's quality of life. Tissue engineering is an emerging interdisciplinary field and innovative healing method with less than three decades of history. It is focused on three main fundamentals such as cells, growth factors (GFs), and scaffolds [5-7]. In bone tissue engineering, scaffolds may form an extracellular matrix (ECM) that can harbor cells, ensure proper biological signaling and facilitate the formation of new tissue scaffolds with suitable and desired topological space. The space in the scaffolds contain cells and able to exchange biological molecules, which may lead to the formation of new hard tissue. The porous scaffold starts to degrade inside the body as the cells planted within the scaffold, then start to grow and reproduce. This may continue until the scaffold is replaced with a new bone tissue to form a structure called a "cell complex" [6-7]. Additionally, there also exist non-cellular based complexes that can be used as scaffolds but using a cell complex in bone tissue engineering was proven to be more effective. While utilizing a non-cellular complex, the scaffold should be able to induce bone growth-stimulating signals. The scaffolds can also contain various GFs and ECM proteins to enhance bone growth stimulation. Considering the importance of quality and healing rate in modern treatment methods, the goal of this research project is to develop a freeze-dried porous nanocomposite scaffold using alginate, hyaluronic acid and titanium dioxide nanoparticles [6-8]. There are different methods to develop scaffolds considering their applications, methods like 3D-printing, space-holder, freeze-drying and electrospinning [9-13, 36]. Discussing the research, taking materials and applications into consideration, the freeze-drying method has been chosen. Titanium dioxide nanoparticles that are less than 50 nm in diameter have been used in order to induce antibacterial characteristics, lowering the risk of infection and accelerating the healing process [14-19]. Presence of nanoparticles in hydrogels may lead to the development of some unique attributes such as responsiveness to mechanical, optical, thermal, sonic, magnetic and electrical stimuli [20-26]. These attributes broaden their usage in many different fields like electronics, optics, sensors, therapeutic stimuli, sorting systems, drug delivery, and medical technology fields [27-38]. Alginate is a natural polymer and has been used for many biomedical applications, due to its biocompatibility, low toxicity, relatively low cost, and mild gelation [39-41]. Hyaluronic acid (HLA) is another component of the nanocomposite structure and it possesses little propensity to cause allergic reactions, speeds up the wound healing process, regulates tissue inflammation levels, improves vessel growth in damaged areas, manages pain relief, having antibacterial properties, and facilitates wound healing in diabetic patients [9-11]. Naturally, HLA is present in the joints as it keeps the bones well lubricated and prevents them from grinding against each other. HLA supplements and injections were proven to be helpful for treating people with osteoarthritis and it significantly reduces pain over time. As the scaffold degrades, the HLA gets absorbed by the body and acts as a drug delivery system (DDS) [12-19]. This scaffold's composition could develop beneficial chemical and biological characteristics. In this research project, four different samples containing various amount of titanium oxide was fabricated using freeze drying technique. Furthermore, their mechanical properties, tensile strength, hardness and wettability were evaluated followed by biological properties including bioactivity, biocompatibility and toxicity.

## MATERIALS AND METHODS

In this research project, the novel alginate-hyaluronic acid filler fabricated using chemical process and freeze-drying technique after discussion with orthopedic surgeons. In this work, starting materials are as following; hyaluronic acid (HLA, 98% purity, Merck company, Germany), alginate (Alginate, 98% purity, Sigma-Aldrich, US), Titanium oxide nanoparticles ($TiO_2$, 50 nm-70 nm, 98% purity, Merck company, Germany), acetic acid ($CH_3COOH$, 99.9% purity, Razi, and Iran) and deionized water. First, 80 ml of deionized water was mixed with 5 g of alginate in a hotplate with 50°C at 500 rpm in order to homogenize the polymeric solution. Then, another 3.75 g of alginate was added and the stirrer set to 60°C at 700 rpm. Next, 2 vol % of acetic acid was diluted with 5 mL of deionized water and added to the primary solution. Furthermore, 10 g of alginate and 8.75 g of hyaluronic acid (HLA) was added to the solution, the stirrer was set to 1000 rpm for 2 hours to become more homogeneous. The magnetic stirrer was set to 70°C at 800 rpm, 3 mL of glutaraldehyde crosslinking agent with chemical formula $C_5H_8O_2$

was added to the solution and divided into three different falcon tube containing 30 mL of solution. Titanium dioxide nanoparticles were added to each Petri dishes and the specified amounts as follows: first sample (S1) containing 0.000 g, second sample (S2) 0.500 g and the third sample (S3) 1.000 g were prepared. Solutions were left on the stirrer at 65°C at 450 rpm for 6 hours to become homogeneous. Then, solutions were poured into the respective Petri dishes and kept at -65°C for 24 hours (DOORSATECH company). The fourth sample (S4) was made multilayered by pouring 10 mL of first solution and second solution into the petri dish, placing it in a freezer for a minute and overlaying it with 10 mL of S1 thereafter. Next, the mixtures were placed on a freeze-drying machine for 24 hours, consisting of an 18-hours main drying process and 6 hours of final drying. After the freeze-drying process was done, the porous filler scaffolds were prepared and cut into pieces for further mechanical and biological evaluations.

*Mechanical and Biological Testing*

Mechanical and biological testing have been executed to ensure the structural rigidity and its capability temporarily replace the damaged bone tissue. First, the tensile strength tests have been performed using a SANTAM-STM50 device in the CRLAB at Amirkabir University of Technology. The tensile strength test measures the maximum amount of tension applied towards the outsides of nanocomposite until it fails. Then, the microhardness tests have been performed using a microhardness device to measure the resistance against plastic deformation. The wettability tests have been performed using a CCD camera at CRLAB, Tehran Polytechnic. The importance of wettability test, is to examine the interactions between nanocomposite's surface and liquids. The biological test such as biodegradation rate, cell adhesion and cell growth in presence of the nanocomposite. As for measuring the nanocomposite's water absorption percentage, samples were weighted before and after putting them into phosphate buffered saline (PBS) for 24 hours. The results were calculated using the following formula:

$$Swelling\,(\%) = \frac{w_2 - w_1}{w_1} \times 100$$

$W_1$ is the dry sample weight and $W_2$ shows the swelled sample weight. Cytotoxicity tests were executed at Tehran Polytechnic, Iran. Firstly, osteoblast cells were acquired from Pasteur institute, Tehran, Iran. Table 1 displays the substances used to prepare the growth medium along their manufacturer and country of origin. Filtered flasks were used for cell culture process and passaging was done every two days until reaching desired cell quantity. Nanocomposite scaffold samples were laid into 70% ethanol for 48 hours and then washed with PBS solution. Cells were counted using a trypan blue solution (0.4g of TP per 100 ml of PBS) and then $3\times10^5$ cells were placed on each sample.

*Phase and morphology analysis*

To characterize the phase and morphology of the bone fillers, the samples were analyzed using X-ray diffraction (XRD) and scanning electron microscopy (SEM) analysis. For preforming XRD test, the three samples powders were analyzed before and after putting them into SBF. The diffraction algorithms gathered from these samples were compared to calcium phosphate related compositions XRD cards, available in (International Center for Diffraction Data) data base, using impact crystal software. Compositions

Table 1: The substances used to prepare cells and to perform cytotoxicity test along their manufacturer and country of origin.

| Substance | Manufacturer | Made in |
|---|---|---|
| RPMI 1640 | Biowest | France |
| Fetal Bovine Serum (FBS) | Biowest | France |
| Phosphate buffered saline (PBS) | Biowest | France |
| Trypsin | Biosera | France |
| Antibiotics (penicillin, streptomycin) | Gibco | United States |
| Dimethyl sulfoxide (DMS) | Gibco | United States |
| (MTT)3-(4,5-dimethyl ethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide | Gibco | United States |
| Glutaraldehyde | Gibco | United States |
| Trypan Blue | Sigma-Aldrich | United States |

and phases within each sample were identified. As for the SEM analysis, it was done using gold coating to analyze the samples.

## RESULTS AND DISCUSSION

Fig. 1 shows a schematic of preparation of alginate-hyaluronic acid containing titanium oxide nanoparticles. Fig. 2 shows the XRD pattern to confirmed the presence of alginate, hyaluronic acid and $TiO_2$ nanoparticles. There are no unwanted compounds or bi-products to be detected. The XRD pattern also indicated that the mineralization degree of alginate hydrogel had been increased by addition of $TiO_2$ and HLA. The SEM images of composite samples are displayed at Figs. 3. By comparing the images related to S1


Fig. 1: Schematic of alginate-hyaluronic acid nanocomposite containing titanium oxide nanoparticles.

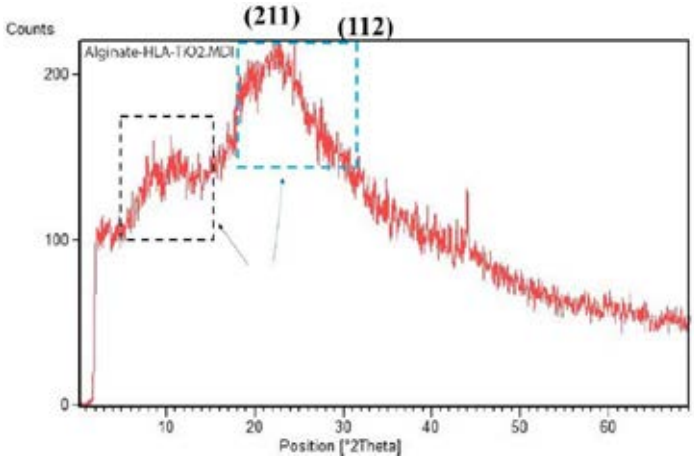


Fig. 2: XRD pattern of XRD pattern indicated that the mineralization degree of hydrogel had been increased by addition of $TiO_2$ and HLA

to others, it showed that the addition of titanium oxide nanoparticles may regulate and decrease the amount of porosity. The porosity percentages can be estimated by inspecting the SEM images. As it is seen in the SEM images, the porosities have a diameter between 100 µm and 200 µm, which shows the spaces between material layers. Fig. 4 (a-b) shows the SEM image of sample 3 with addition of $TiO_2$ and HLA in which the $TiO_2$ nanoparticles have agglomerated arrangement with 20-40 micron size.

Fig. 5 reveals that porosities resemble a honeycomb microstructure which the porous are connected to each other. The amount of space needed for bone tissue cells to grow and populate is around 100 µm to 250 µm in diameter, which the produced scaffold is ascertained to provide. By investigating the images from samples that contain nanoparticles, it is clear that the nanoparticles are well merged within the composite's matrix and no improper interaction can be reported. The aggregation of titanium oxide nanoparticles in

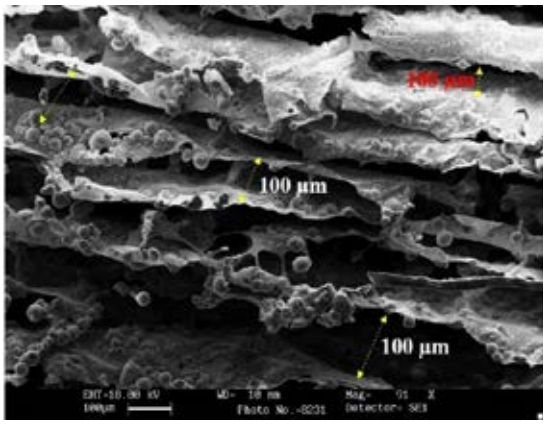


Fig. 3: SEM images filler scaffold prepared using freeze drying technique.

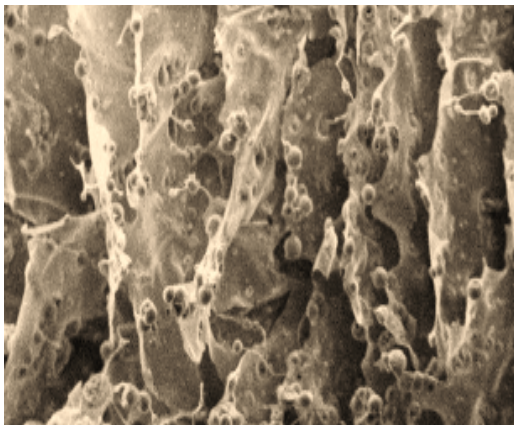

Fig. 4: SEM image of S3 composite hydrogel with addition of $TiO_2$ and HLA

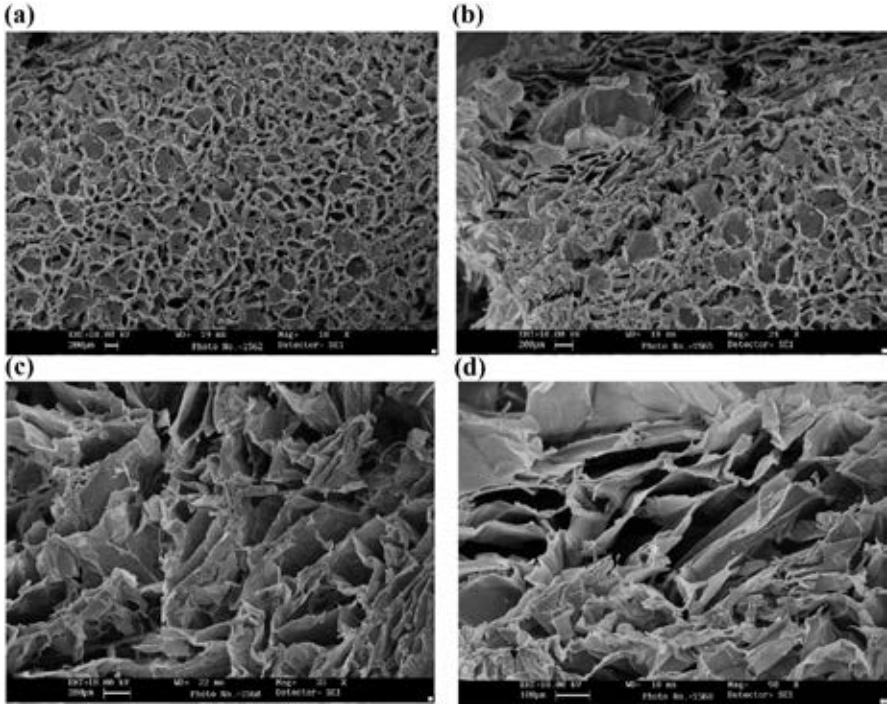


Fig. 5: SEM image of composite hydrogel compared to S1, caused by the addition of TiO2 (a) S2, (b) S2, (c) S1 and (d) S1

some areas are caused by the gravitation between oxygen in $TiO_2$ and HA's acidic agents, which may form a hydrogen bond together. Taking these factors into account, this could be the cause of a better tensile strength performance in samples containing nanoparticles. This could also result in a more predictable biodegradation, smaller degraded pieces and reduce pH changes in the area of application. It could be understood from the images that freeze-drying method worked out well, considering materials and composite's purpose, by forming porosities with good dimensions and distribution.

As shown in Figs. 6 and Fig. 7, the addition of titanium oxide nanoparticles has a linear relation with the enhancement of tensile strength results of the samples. The obtained results of S4, which is a multilayered sample consisting of S1 and S3, improved very little compared to S3 which shows its dependent on its S3 layer concerning tensile strength. In addition, a multilayered composite design does not support that much in this manner. As shown in Fig. 8, there is a slight improvement between S1 and S2 and also between S3 and S4 microhardness test results, which indicates that small amounts of titanium oxide nanoparticles and multilayered design does not support improving mechanical results. Fig. 9 projects the wettability test results of all four samples. There is a little improvement from S1 to S2 but with the addition

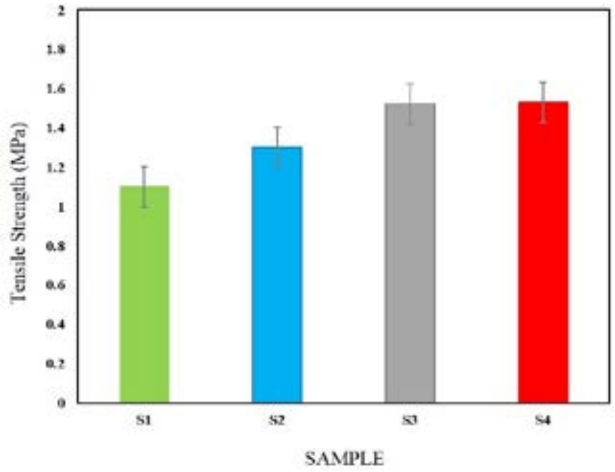


Fig. 6: Tensile strength results of nanocomposite scaffolds, executed following measurements are as the following; S1:1.1, S2:1.3, S3:1.52 and S4:1.53.

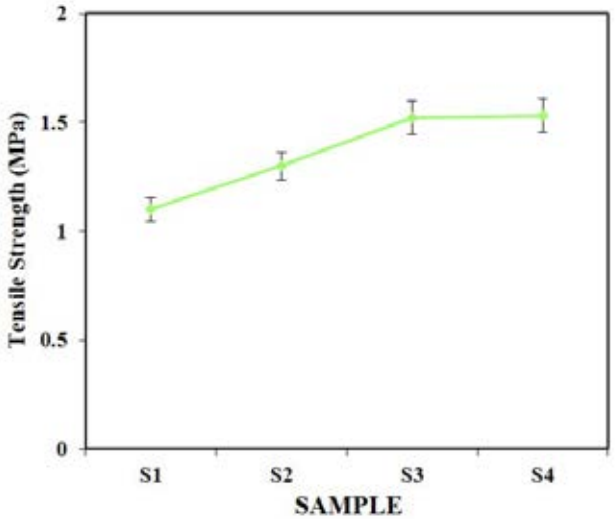


Fig. 7: Tensile strength result of hydrogel filler for bone trauma application evaluated using SANTAM-STM50 machine.

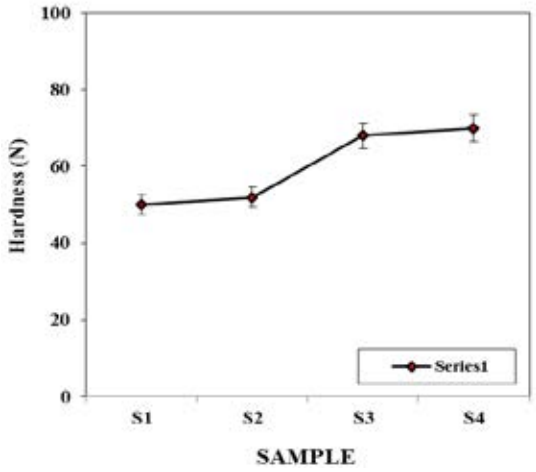


Fig. 8: Microhardness test results of nanocomposite scaffolds, executed following hardness test, The exact measurements are as the following; S1:50, S2:52, S3:68 and S4:70.

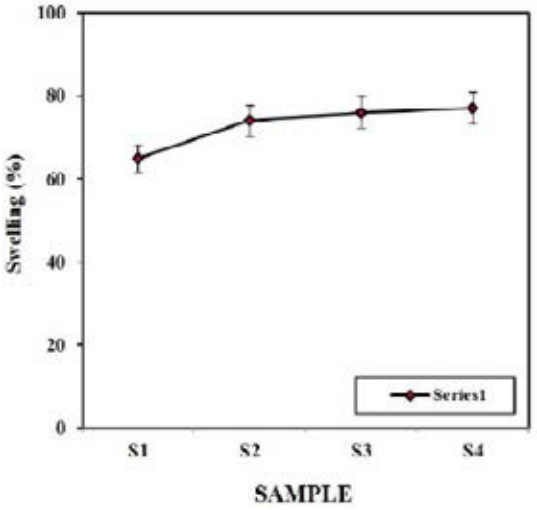


Fig. 10: Water absorption (swelling) test results of nanocomposite scaffolds. The exact measurements are as the following; S1:65, S2:74, S3:76 and S4:77.

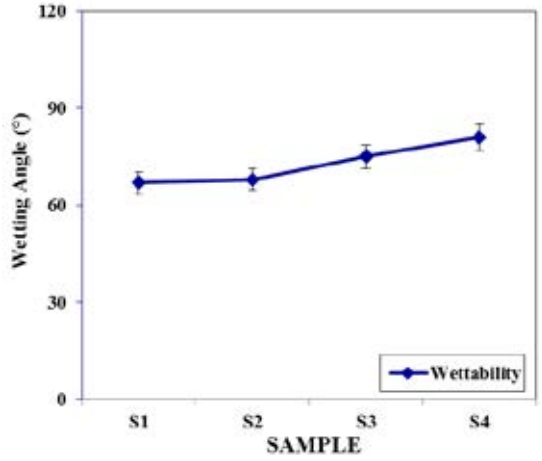


Fig. 9: Wettability degree test results of nanocomposite scaffolds measurements are as the following; S1:67, S2:68, S3:75 and S4:81.

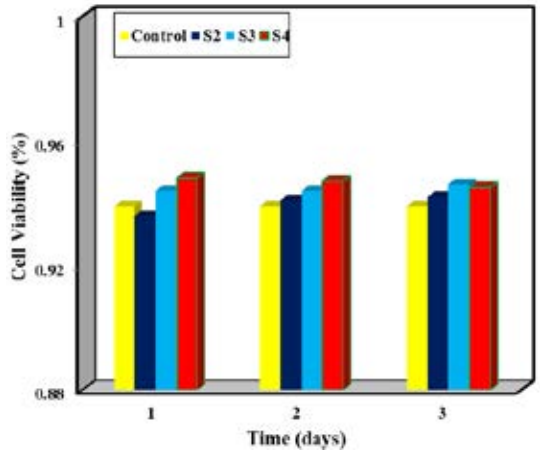


Fig. 11: Fibroblast and cell culture evaluation of alginate-hyaluronic acid nanocomposite scaffolds.

of nanoparticles there is an amplification of wetting angle, as seen in S3. The fascinating part is the S4's wettability degree increases from S3. As the sample with multilayered structure consisting of S1 and S3, it was expected that results may be between the S3 and S1 results regarding the wettability degree, however; the results showed vice versa. The wettability results enhanced notably because of the multilayered structure. The $TiO_2$ nanoparticles cause the samples to have less hydrophilic property while it may have a controlled biodegradation and cell anchoring. Water absorption was measured by weighting the scaffolds before and after inserting them into PBS for 24 hours as shown in Fig. 10. By comparing the numbers, it can say that as $TiO_2$ nanoparticles amounts increase, the water absorption increases too but not as much as the initial addition of $TiO_2$ to S2. This increase in water absorption could be occurred by two factors. The first factor is the increase in composite's structural polarity caused by the addition of $TiO_2$, and the water present in PBS solution has a polar molecule as well, so the more polar the composite is, the more it attracts water. The second factor, as stated by SEM images, is the improvement of composite's porosity and morphology by the addition of $TiO_2$ nanoparticles, which may lead to a better permeability. Cytotoxicity test results are shown

Table 2: The cytotoxicity test results of the fabricated hydrogel composite, displaying cell viability values.

| Sample | Cell viability (%) Day 1 | Cell viability (%) Day 2 | Cell viability (%) Day 3 |
|---|---|---|---|
| Control Sample | 93.9 | 93.9 | 93.9 |
| Sample 2 | 93.6 | 94.1 | 94.2 |
| Sample 3 | 94.4 | 94.4 | 94.6 |
| Sample 4 | 94.8 | 94.7 | 94.5 |

in Fig. 11 and Table 2. Presence of nanocomposite samples improved osteoblast cell growth and viability compared to the control sample. It can be understood that the nanocomposite's biodegradation species are not toxic to the cells but rather beneficial to them. At first S2 had the lowest viability with 93.6% but its results improved in the following days. The S3 sample, which has the most amount of $TiO_2$ nanoparticles, had a better cell viability than S2 with 94.4%. S3's cell viability stayed the same for the second day but as of the third day it improved to a 94.6% cell viability, which was the best result of the last day. The MTT results for the sample 4 show a best initial viability with 94.8% but it decreased to 94.5% in following days. This decline in cell viability results could be a result of weaker S1 layer performance in porosity and water absorption fields.

## CONCLUSION

This project succeeded in developing nanocmposite samples based on natural alginate and hyaluronic acid hydrogel polymers reinforcing with titanium oxide nanoparticles. Freeze-drying technique. The analysis of mechanical properties and biological evaluations proved the addition of $TiO_2$ nanoparticles improved the tensile strength results. There was a linear relation between the addition of $TiO_2$ nanoparticles amount and tensile strength improvement. The S4's layered design can support very similar to sample of S3's. The microhardness test result showed a small amount of $TiO_2$ nanoparticles may affect the mechanical performance results significantly. Wetting angle stayed in the preferable spectrum, the composites are hydrophilic but not too much and can provide a good cell adhesion in contact with the cells. The addition of $TiO_2$ nanoparticles increased the wetting degree and S4's layered design was as effective as nanoparticles. Furthermore, analyzing phasic and morphologic evaluations, XRD pattern showed no signs of unwanted bi-products and compounds. The SEM images showed that the addition of $TiO_2$ nanoparticles enhanced the samples porosity form and structure distribution. Measuring the porosities dimensions showed composites are suitable for cell growth with the size that cells can growth properly. Water absorption test also had favorable results, better swelling results mean better cell nutrition and growth, thus $TiO_2$ nanoparticles proved to be beneficial. Cytotoxicity test results showed enhancement in cell viability except S4. The sample 4 started with the best cell viability on the first day but its viability decreased in the following days of the test, therefore sample 3 had better cell viability compared to the other samples. Also, there was no unnatural and concerning cell growth and viability. Taking all tests and evaluations into account, S3 proved to have the best overall performance among other nanocomposite samples. S4 had better mechanical characteristics but its cytotoxicity result was not favorable. It is true to say adding $TiO_2$ nanoparticles was beneficial in every way but further investigations are advised. Physically and biologically investigation, injectable scaffolds are more advantageous because of their less invasive application. HLA-$TiO_2$ prepared using freeze drying technique is a scaffold which can produce a super porous injectable filler with a specific form. As this project aims to mimic natural bone tissue, the chosen materials can also be used as a cryogen.

## ETHICAL APPROVAL

This study started after receiving its scientific ethical approval from Isfahan University of Medical Sciences with evaluation of experimental and comparison investigation of bone filler that registered inquiry and funding under the No. 399163 and IR.MUI.RESEARCH.REC.1399.292.

## CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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