



Article

Chitosan–Hydroxyapatite Composite Chemical/Physical Crosslinking Scaffolds for Cell Cultivation

Yuliya Nashchekina ^{1,*}, Yuri Novosad ² , Elena M. Ivan'kova ^{3,4} and Vladimir Yudin ^{3,5}

- ¹ Institute of Cytology of the Russian Academy of Sciences, Center of Cell Technologies, Tikhoretsky Pr. 4, Saint Petersburg 194064, Russia
- ² H. Turner National Medical Research Center for Children's Orthopedics and Trauma Surgery, Parkovaya 64–68, Pushkin, Saint Petersburg 196603, Russia; novosad.yur@yandex.ru
- ³ Branch of Saint Petersburg Nuclear Physics Institute Named by B.P.Konstantinov of National Research Centre «Kurchatov Institute»—Institute of Macromolecular Compounds, V.O., Bol'shoy Pr. 31, Saint Petersburg 199004, Russia; ivelen@mail.ru (E.M.I.); yudinve@gmail.com (V.Y.)
- ⁴ S. M. Kirov Military Medical Academy, Academician Lebedeva Str., 6G, Saint Petersburg 194044, Russia
- ⁵ Higher School of Biomedical Systems and Technologies, Peter the Great St. Petersburg Polytechnic University, Polytechnicheskaya St. 29, Saint Petersburg 195251, Russia
- * Correspondence: nashchekina.yu@mail.ru

Abstract

The development of biocompatible and mechanically flexible skeletal scaffolds is a significant challenge in modern regenerative medicine. In this study, we developed composite scaffolds based on biodegradable chitosan polymer and hydroxyapatite particles. We have shown for the first time that treatment with sodium hydroxide solution, which is often used to convert chitosan scaffolds into an insoluble form, can cause alkali sorption by hydroxyapatite particles. This has been demonstrated by our experiments. It has also been shown that the alkaline treatment of composite scaffolds increases the pH of the surrounding culture medium, reducing the viability of mesenchymal stromal cells by 60–70%. As an alternative to the processing of composite scaffolds using chitosan and hydroxyapatite, we propose heat treatment. This method allows us to produce stable scaffolds without affecting cell viability. Heat treatment promotes the formation of bonds between free amino groups in chitosan and phosphate groups in hydroxyapatite, as well as increasing the elasticity of the composite matrices in humid conditions.

Keywords: chitosan; hydroxyapatite; alkaline treatment; temperature treatment; mesenchymal stromal cells



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1. Introduction

Bone tissue damage is one of the most common injuries and is accompanied by loss of motor activity, individual disability and, as a result, expensive treatment [1]. Restoration of damaged bone tissue is one of the urgent tasks of modern regenerative medicine and tissue engineering. The effectiveness of rapid healing and formation of new bone tissue depends on the properties of a tissue-engineered structure, which is a biocompatible polymer scaffold (carrier) with cells. Different materials are used to create scaffolds for use in the regenerative engineering of bone tissue, such as polylactides [2,3], copolymers based on lactide and caprolactone [4,5]. Polymers based on natural materials, such as collagen [6,7] and silkfibroin [8], are great interest for bone tissue engineering. Thus, the high demand for such biomaterials is beyond doubt. For the successful use of biomaterials in tissue engineering of bone tissue, they must have not only high biocompatibility and

characteristic structure, but also sufficiently high mechanical strength to withstand loads during operation [9].

Chitosan is widely used in tissue engineering because of its availability, antimicrobial activity [10], non-toxicity and high-adsorption properties [11]. Chitosan has good biocompatibility and is decomposed by enzymes present in the human body [12,13]. Its uniqueness is due to the structure of the molecule: the presence of links in the composition of the polysaccharide, which has a number of chemical and structural similarities with collagen [14]. Additional advantages of chitosan in comparison with other polymers are its ability to stimulate natural blood clotting and the formation of an anti-infective barrier [15–18].

Despite a large number of advantages, chitosan scaffolds have relatively low mechanical strength and cannot carry significant mechanical loads inherent in bone tissue. Nevertheless, the mechanical properties of chitosan can be improved by obtaining composite scaffolds with hydroxyapatite (HA).

Use of HA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a reasonable choice when developing new methods of bone treatment, because it is the main component of the hard tissues of vertebrates and makes up 60–70% of the bone mass and 98% of the tooth enamel mass. The use of hydroxyapatite for successful bone regeneration is due to the similarity between the elemental composition of the bone matrix and synthetic hydroxyapatite. In addition, modern techniques allow for the synthesis of hydroxyapatite with a crystal structure that closely resembles the crystal structure of human bone [19,20]. Due to its similarity to the natural mineral phase of human bone, artificial HA is widely used in biomedicine for bone repair and bone tissue regeneration, where osteoconductivity is considered a key property [21]. On the basis of HA, both inorganic scaffolds and composite ones are obtained in combination with biopolymers [22]. Despite the fact that inorganic scaffolds from HA have optimal morphology and porosity, they are usually characterized by insufficient mechanical properties. For this reason, in most cases, composite scaffolds based on natural polymers in combination with HA are used for tissue engineering of bone tissue [22].

The successful use of composite scaffolds based on chitosan and HA obtained by various methods in the treatment of various bone defects has been demonstrated in many literary sources [23,24]. In the process of obtaining chitosan scaffolds, regardless of the shape of the product (films, fibers, porous scaffolds), it is necessary to convert the water-soluble salt form of chitosan into an insoluble form [25]. Deacetylation of chitosan salts with sodium hydroxide solution is an effective and fast way to convert the polymer into a water-insoluble form [26]. This method is fast and fairly effective, and it is generally available. Most authors use this method for processing chitosan scaffolds. Before, these scaffolds were rinsed thoroughly with water after alkali treatment to remove any residual alkali. However, when using fillers, especially porous ones, it is important to remember that they can absorb alkali during the formation of composite scaffolds. This can lead to the alkali not leaving the porous structure completely. When preparing composite frameworks, it is important to take into account the interaction not only between sodium hydroxide and chitosan, but also between sodium hydroxide and other components. We analyzed papers and found that this question was not addressed in them. For the first time, we showed in our study that HA particles in a composite with chitosan can interact with sodium hydroxide, absorbing alkali that can crystallize inside the HA particles. Such sorption of sodium hydroxide by hydroxyapatite particles can lead to a toxic effect on the composite scaffold, despite the high biocompatibility of the initial components, such as chitosan and hydroxyapatite. Therefore, to obtain a biocompatible framework, it is necessary to carefully control the complete removal of alkali from the final product. Alternative methods for crosslinking chitosan and converting it into an insoluble form can help solve this problem.

At the same time, it is desirable that the alternative method does not involve the use of chemical agents that could be sorbed onto the porous hydroxyapatite surface, but rather a physical method that would not alter the structure of the original components. An alternative way to convert the salt form of chitosan into a form insoluble in water is heat treatment [27,28]. According to data from the literature, the decomposition of chitosan salts occurs during thermomodification, followed by the amidation of chitosan by the released acids, which leads to a change in the crystal structure and crosslinking of the polymer [29]. The aim of this study was to obtain thermomodified biocompatible composite scaffolds based on chitosan and HA and compare their properties with composite scaffolds treated with sodium hydroxide solutions.

2. Materials and Methods

2.1. Materials

BiologHeppe chitosan (Landsberg, Germany) with a molecular weight of 164 kDa and a deacetylation degree of 92.5% was used to prepare scaffolds.

The HA was obtained according to a previously published methodology [30]. The synthesis was carried out from aqueous solutions of ammonium hydrophosphate $((\text{NH}_4)_2\text{HPO}_4)$ and calcium nitrate $(\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O})$ in distilled water. Salt solutions were used with a molar ratio of $\text{Ca}/\text{P} = 1.67$, which coincides with the ratio of biogenic hydroxyapatite in bone tissue. A solution of ammonium hydrophosphate and calcium nitrate was mixed with constant stirring; the pH of the resulting suspension was 5.0–5.1. Then the suspension was transferred to a Teflon crucible with a volume of 100 mL and hydrothermal treatment was carried out at $T = 200\text{ }^\circ\text{C}$ and a pressure of 10 MPa for 24 h. After that, the obtained samples were washed with distilled water until $\text{pH} = 7$ was reached and dried at $T = 50\text{ }^\circ\text{C}$ in an air atmosphere.

2.2. Mesenchymal Stem Cells

A culture of rabbit mesenchymal stem cells isolated from the animal's adipose tissue was used in the work. The cells were cultured in a nutrient medium, alpha-MEM (PanEco, Moscow, Russia), with penicillin and the addition of L-glutamine, 10% bovine embryonic serum (PanEco, Moscow, Russia) and antibiotics (100 units/mL of penicillin, 100 micrograms/mL of streptomycin (Gibco, Grand Island, NY, USA)). Cells of 5–9 passages were used in the work.

2.3. Scaffold Preparation

To obtain the scaffolds, chitosan was dispersed in distilled water for 15 min until it swelled. Then glacial acetic acid (Lenreactive, Saint Petersburg, Russia) was added to the resulting dispersion to a final acid concentration of 2%. The obtained mixture was mixed until the chitosan was completely dissolved.

When obtaining the composite scaffolds, HA was dispersed on an IL-10 ultrasonic dispersant (Russia) for 30 min at a frequency of 24.5 GHz to obtain nanosized apatite crystals and then added to the resulting chitosan solution and stirred for 30 min to evenly distribute the particles in the chitosan solution.

Further, chitosan solutions with HA particles and without particles were cast into molds and frozen at a temperature of $-18\text{ }^\circ\text{C}$ for 12 h. Freeze-drying was carried out at a pressure of 4 Pa and a temperature of $0\text{ }^\circ\text{C}$ for 24 h (Labconco Free Zone Triad 2.5 L, Labconco, Kansas City, MO, USA).

Two methods were used to convert chitosan scaffolds after lyophilic drying into an insoluble form:

Alkaline method: Finished scaffolds were treated with 10% sodium hydroxide (NaOH) solution for 30 min. To remove the alkali, the scaffolds were washed twice in distilled water for an hour.

Thermal method: Chitosan scaffolds were placed in a thermostat at a temperature of 100 °C for 4 h.

For treatment of HA particles in acid/alkali solution, 1 g of HA particles was dispersed in distilled water or 1% acetic acid solution/1% sodium hydroxide solution. Precipitation of the particles after incubation in the test solution was carried out by centrifugation for 5 min. After that, the HA particles were boiled at a temperature of 100 °C for 1 h to remove acetic acid and alkali residues in the samples. Then the particles were washed 5 times in distilled water for 2.5 h.

The particles were sterilized by autoclaving at a temperature of 121 °C and at a pressure of 0.11 MPa for 1 h.

2.4. pH Metric

After thorough washing, the HA particles were incubated in water for 2 weeks. The pH measurement was carried out using a pH meter pH-150MI (Measuring Equipment LLC, Moscow, Russia).

2.5. Scanning Electron Microscopy

The structure of native HA particles, particles after acid and alkaline incubation, as well as the structure of chitosan scaffolds with and without HA particles converted to an insoluble form in various ways were visualized using scanning electron microscopy (Supra 55 VP (CarlZeiss, Oberkochen, Germany)). Previously, a layer of Pt with a thickness of ~11 nm was sputtered on the sample surface with the help of a sputtering device, Q150T ES (Quorum Instruments, Lewes, Great Britain).

2.6. FTIR Spectroscopy

Chitosan scaffolds before and after conversion to insoluble form were analyzed using a Fourier transform infrared spectrometer (FTIR), IR Prestige-21 (Shimadzu, Tokyo, Japan), in reflection mode with a resolution of 2 cm⁻¹.

2.7. Mechanical Characteristics

The effect of HA, as well as incubation in alkali and heat treatment on the mechanical properties of the scaffolds, was evaluated using an Instron 5943 universal testing machine (UK) in the sample compression mode. The study was carried out at room temperature with a compression rate of 0.5 mm/min. The samples in the form of a parallelepiped with dimensions of 15 × 15 mm² and a height of about 7 mm were used for analysis. To study the mechanical characteristics of the chitosan scaffolds in the wet state, the samples were incubated in water for a day.

2.8. In Vitro Studies

To assess the toxic effect of the HA particles and chitosan scaffolds obtained by various methods, the MTT method was used. Sterile HA particles were incubated in a complete nutrient medium (alpha-MEM, 10% bovine serum, L-glutamine and streptomycin) for 1 week. The nutrient medium was selected after 1, 3 and 7 days of incubation and sterilization.

Chitosan scaffolds were sterilized by autoclaving at a temperature of 120 °C at a pressure of 0.1 MPa within 1 h. After sterilization, the scaffolds were incubated in the complete nutrient medium in a CO₂ atmosphere at a temperature of 37 °C for 7 days.

Mesenchymal stromal cells were applied to a 96-well plate, with 5 thousand cells per well. After the cells were fully attached for 1 day, the medium was removed and the

conditioned medium was added to the cells after incubation with the full nutrient medium. As a control, cells cultured in the standard nutrient medium and extracts of the chitosan matrices were used and cultured for three days. Next, the nutrient medium was removed from the wells and a mixture of 5 µL of 3-(4,5-dimethazole-2-yl)-2,5-diphenyl tetrazolium bromide (Thermo Fisher Scientific, Waltham, MA, USA) was added with 45 µL of the nutrient medium and incubated at 37 °C in 5% CO₂ for 2 h. During incubation, the yellow MTT solution was transformed into blue–violet formazane crystals under the action of mitochondrial dehydrogenase, which is present in living cells. The formed formazane crystals were dissolved in 50 µL of dimethyl sulfoxide (DMSO, Paneco Ltd., Moscow, Russia). The optical density of the formazane solution was studied at a wavelength of 570 nm on a SPECTROstar® flatbed spectrophotometerNano (BMG LABTECH, Ortenberg, Germany).

2.9. Statistical Processing of Results

Due to the small sample size, the data distribution was considered to deviate from normality. Nevertheless, given equal group sizes, a one-way analysis of variance (ANOVA) was applied to assess differences among groups. The ANOVA revealed statistically significant differences between groups ($p < 0.001$). Multiple post hoc comparisons were performed using Tukey's HSD test. All experimental groups were found to be statistically significantly different from the control group (** $p < 0.001$; **** $p < 0.0001$).

3. Results and Discussion

3.1. Analysis of HA Particles After Treatment with Acetic Acid and Alkali Solutions

HA is actively used as a filler for bone defects, as well as in composite scaffolds, including in combination with chitosan. An analysis of publications over the past 10 years has shown that the main precipitator of chitosan for converting it into an insoluble form when obtaining scaffolds for cell cultivation and transplantation is alkali, most often sodium hydroxide [31–33]. It should be noted that most studies demonstrate a positive effect of the presence of HA particles not only on the mechanical properties of the composite scaffolds, but also on the osteoinductive properties of the obtained scaffolds both during cell culture and after their implantation into a bone defect [34]. It has been reported that HA particles exhibit good adsorption and absorption capabilities due to their high porosity [35,36]. During the process of obtaining a composite chitosan scaffold, chitosan is dissolved in acetic acid and then treated with an alkali to convert it into an insoluble form. Both acid and alkali come into contact with the HA particles, potentially affecting their properties during the creation of the composite scaffold. To confirm this hypothesis, the HA particles were incubated in a solution of acetic acid and alkali. HA particles served as a control group after being incubated in water, to eliminate the influence of hydrolysis on their properties. Afterwards, the particles were rinsed thoroughly with water to remove any acid or alkali residue. The structure of the HA particles was analyzed using SEM after acid and alkali treatment. The findings of these investigations are presented in Figure 1A.

Figure 1A shows that the GC had a microporous structure. According to the scanning electron microscopy (SEM) data, there were no significant structural differences between the HA particles incubated in water and those incubated in an acetic acid solution. The HA particles had a more spherical shape, and the particle size had a polydisperse distribution, ranging from a few microns to 10 microns. Additionally, the SEM images show that the HA particles aggregated into conglomerates of various sizes, ranging from several tens of microns in diameter. All of these particles had a microporous structure, and it should be noted that, after acid treatment, the HA particles could also form elongated cylindrical aggregates measuring 10–15 microns, in addition to spherical aggregates. However, most of the aggregates retained their spherical shape and microporous structure. When HA particles

were treated with an alkaline solution, their morphology changed significantly. Most HA particles aggregated, forming rod-shaped structures 10–20 microns in size, characteristic of crystalline alkaline solutions (black arrows). These structures appeared on the surface and between the particles. However, the microporous morphology of the HA particles was preserved. Probably, the main cause of the toxic effects of the composite scaffolds was alkali crystallization on the particles, which could change the pH of the nutrient medium.

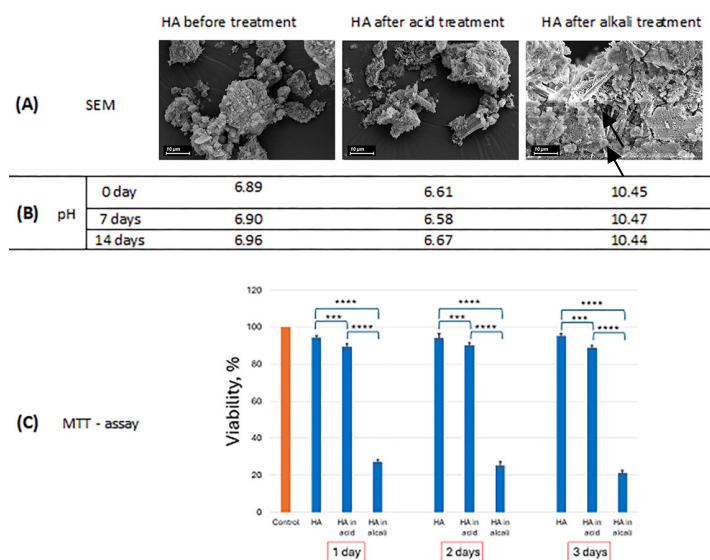


Figure 1. The analysis of the results of the HA particles after incubation in water, acid, and alkali obtained by scanning electron microscopy (A), pH-metry (B), and an MTT test (C).*** $p < 0.001$; **** $p < 0.0001$.

It is known that for normal cell function in culture, the pH of the nutrient medium should be within the physiological range of 7.2–7.4 [37]. A change in pH to either the acid or alkaline side can negatively affect cell viability in vitro [38]. The pH data (Figure 1B) confirmed the findings of the SEM. Even after thoroughly washing the HA particles that were incubated in an alkaline solution, the pH of the medium remained high and reached 10, which could negatively affect cell growth. To confirm this assumption, the conditioned medium after incubation of the HA particles was used for the cultivation of MSCs (Figure 1C). The MTT analysis results showed that the viability of MSCs grown in a conditioned medium after being incubated in water and acetic acid solutions did not differ from the control sample grown in the standard complete nutrient medium. However, the viability of cells cultured in the conditioned medium for 1 day in an alkaline solution significantly decreased compared to that of the control. It is important to note that the number of viable cells after one week of culture in the nutrient medium was only 20% of the control sample. This suggests that the alkaline solution was not only adsorbed on the surface of HA particles, but it also penetrated them, where it crystallized and slowly dissolved before exiting into the nutrient medium. Unfortunately, it is currently not possible to fully control and predict the process of alkali sorption into HA particles and their dissolution during cell culture.

An alternative to the alkaline treatment of the composite chitosan scaffolds containing the HA particles is exposure to heat [28,39]. Although this method of converting chitosan into a water-insoluble form is available, it is rarely used [40]. This method for stabilizing chitosan was first proposed several decades ago but has not been widely adopted. Typically, chitosan is transformed into an insoluble form as films in this process. In this study, we propose for the first time the production of the insoluble composite chitosan porous scaffolds. The influence of the presence of the HA particles on the structure of the resulting

chitosan scaffolds after freeze-drying was assessed using SEM (Figure 2). Our initial studies have indicated that the optimal proportion of the HA particles in the composite matrix for bone grafting was 50% by weight of chitosan [41].

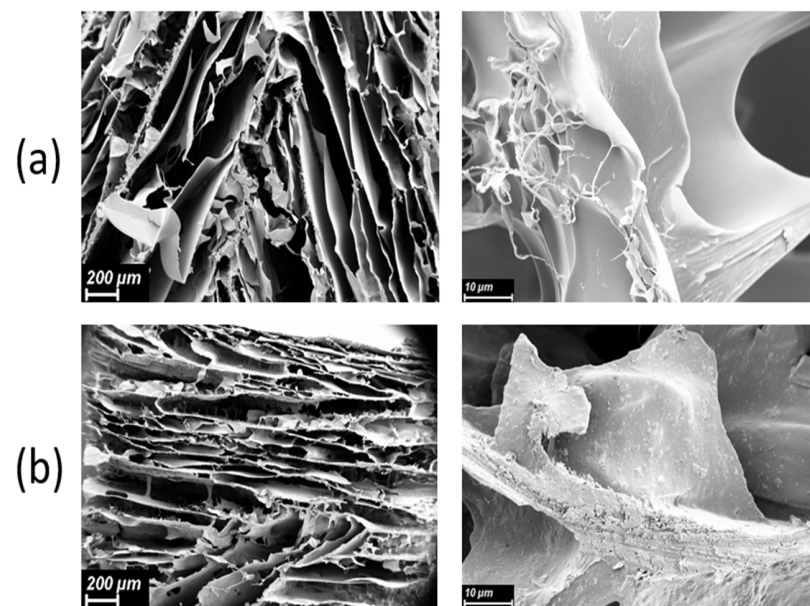


Figure 2. SEM images of the cross-sections of the freeze-dried scaffolds with different magnifications. (a) Chitosan without HA; (b) chitosan with nanosized HA powder.

According to the SEM data, it can be seen that the presence of the HA particles in the composite scaffold, even at 50%, did not significantly affect the overall structure of the composite scaffold (Figure 2). Pores had an elongated shape (Figure 2a), similar to that of chitosan scaffolds [42]. It should be noted that the presence of HA particles in the composite scaffold contributed to a denser structure (Figure 2b). However, the pore size of the composite scaffold was slightly smaller than that of the control sample made from unfilled chitosan. The average pore size in the composite scaffold was about 500 μm, which was large enough for cells to penetrate and migrate into the porous structure [43]. SEM data showed a uniform distribution of the HA particles throughout the chitosan scaffold. This method of production did not result in significant aggregation of the HA particles. Instead, they were evenly distributed throughout the matrix (Figure 2b). After freeze-drying, both composite chitosan scaffolds and scaffolds based on unfilled chitosan were converted to an insoluble form, both by standard treatment with an alkali solution and by exposure to heat. The structure of the resulting scaffolds was analyzed using SEM (Figure 3).

When analyzing the structure of the chitosan scaffolds, it can be observed that treatment of freeze-dried chitosan samples with an alkaline solution led to a change in the scaffold's structure, specifically, a decrease in pore size and a flattening of their shape (Figure 3a,b). This change in the structure is typical for both samples based on unfilled chitosan and those containing the HA particles. Reducing the pore size may be one of the reasons why it was difficult to completely remove the alkali after transferring composite scaffolds into an insoluble form. The structure of the scaffolds, whether they contained the HA particles or not, did not change after heat treatment and remained the same as that immediately after freeze-drying (Figure 3c,d). Pore size remained at 500 microns. The distribution of the HA particles in the chitosan scaffold before and after converting it into a water-insoluble form using chemical and physical methods did not change significantly (Figure 3b,d). The HA particles were evenly dispersed throughout the entire volume of the scaffold.

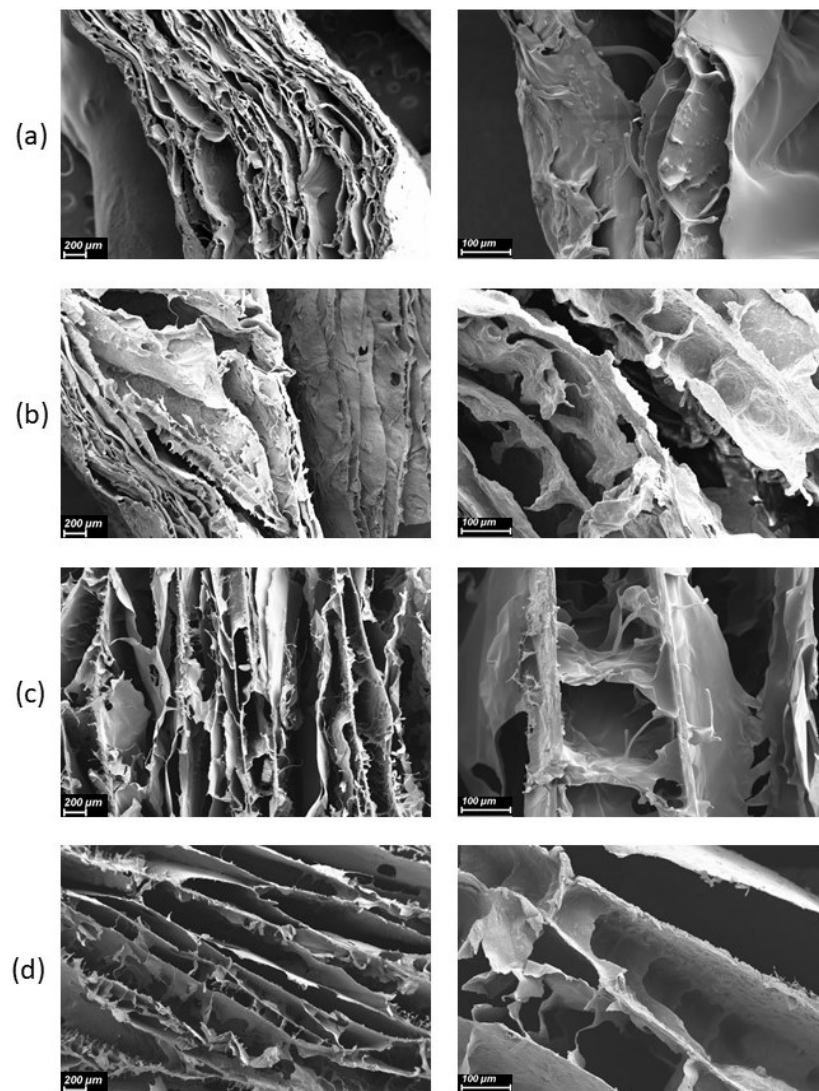


Figure 3. SEM images of the scaffolds with different insolubilization method. (a) Chitosan scaffolds after alkaline treatment (CH-A); (b) chitosan scaffolds with HA after alkaline treatment (CS-HA-A); (c) chitosan scaffolds after temperature treatment (CS-T); (d) chitosan scaffolds with HA after temperature treatment (CS-HA-T). Scale bar corresponding 100 μm .

3.2. Structure of Composite Scaffolds

The IR spectra of the scaffolds obtained from the solution in acetic acid, both with and without the HA, as well as those treated at a temperature with the alkali solution and subsequently aged, are presented in Figure 4. In the initial spectrum of the scaffold without the HA (red), there are two characteristic absorption bands at 1635 and 1320 cm^{-1} , corresponding to the Amide I and Amide III bands, respectively [44,45]. There is also the third band between 1550 and 1560 cm^{-1} corresponding to Amide II. Additionally, there are three more bands between 1530 and 1590 cm^{-1} , which correspond to protonated groups and acetate counterions, respectively. Finally, there is an absorption band at 1404 cm^{-1} that indicates the presence of acetate counterions.

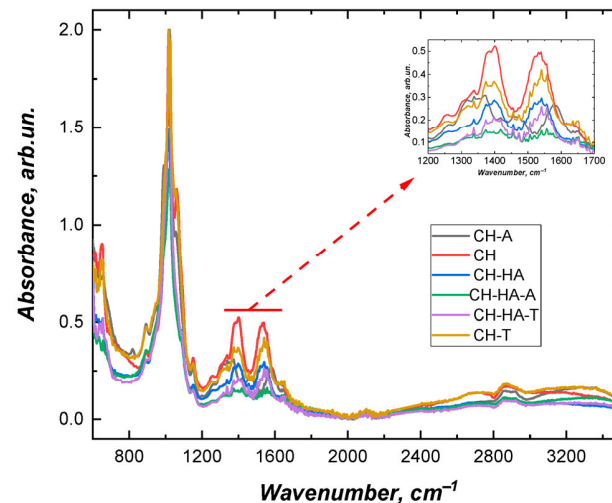


Figure 4. IR Fourier spectroscopy of chitosan scaffolds. CH-A—chitosan scaffolds after alkaline treatment; CH—chitosan scaffolds before alkaline treatment; CH-HA—chitosan scaffolds with HA before alkaline treatment; CH-HA-A—chitosan scaffolds with HA after alkaline treatment; CH-HA-T—chitosan scaffolds with HA after temperature treatment; CH-T—chitosan scaffolds after temperature treatment.

For the scaffolds treated with both alkali and heat, a decrease in signal at 1404 cm^{-1} is observed, suggesting the removal of acetate counterions due to these treatments. A decrease in the intensity of the band at 1541 cm^{-1} for the samples treated with alkali and heat confirms the removal of ionized acidic residues (yellow, black, and purple) [46].

3.3. Mechanical Properties

The mechanical properties of the scaffolds, including their Young modulus, are an important consideration for the scaffolds developed for bone grafting [18]. Figure 5 shows data on the Young modulus of the scaffolds obtained in both dry and wet conditions. The figure demonstrates that the addition of the HA particles to the chitosan scaffolds increased their rigidity, which is in accordance with data from the literature [47]. The increase in rigidity was due to the reinforcement of the chitosan scaffolds with the HA particles. Additionally, it should be noted that the difference in Young's modulus between the chitosan scaffolds with and without HA was greater than twice when the scaffolds were in a wet state. This difference in modulus was also observed for the scaffolds treated with alkali. Meanwhile for the scaffolds obtained under the influence of temperature, there was a significant difference in the modulus of elasticity between the scaffolds without the HA particles and those with the HA particles. This difference was almost five times. Such a substantial increase in the elastic properties of the scaffolds upon addition of the HA particles was due not only to their reinforcing effect, but also to the formation of cross-linking chains between chitosan and HA due to the interaction between chitosan amino groups and Ca^{2+} ions and $(\text{PO}_4)^{-}$ ions from HA [48,49].

It should also be noted that the mechanical properties of the chitosan scaffolds in their wet state after heat treatment are inferior to those of chitosan after alkali treatment. Based on data from the literature and our own research, the conversion efficiency of chitosan to its saline form is greater after alkali treatment compared to heat treatment [50]. However, after heat treatment, there are more free amino groups remaining, which contributes to an increase in the chitosan's binding to the HA particles and, consequently, a more significant increase in Young's modulus, as we observed in our studies.

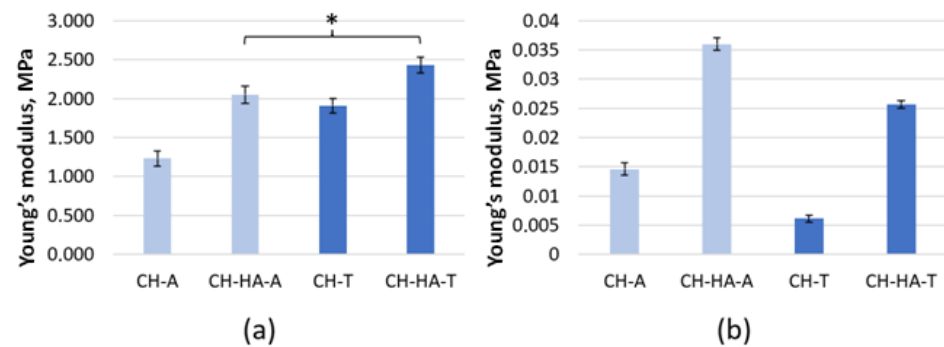


Figure 5. Young's modulus, MPa. (a) Dry scaffolds; (b) wet scaffolds. CH-A—chitosan scaffolds after alkaline treatment; CH-HA-A—chitosan scaffolds with HA after alkaline treatment; CH-T—chitosan scaffolds after temperature treatment; CH-HA-T—chitosan scaffolds with HA after temperature treatment. * $p < 0.05$.

3.4. MTT-Test

The biocompatibility of the obtained scaffolds was evaluated using the MTT test. The composite scaffolds, as well as the scaffolds based on unfilled chitosan, were incubated in the complete nutrient medium in the same way as in the experiment with the HA particles. The conditioned medium after incubation of the scaffolds in the complete nutrient medium was collected after 1, 3, and 7 days. MSCs were then incubated in this conditioned medium for 3 days. After this time period, the morphology of the cultured cells was analyzed using optical microscopy (Figure 6a).

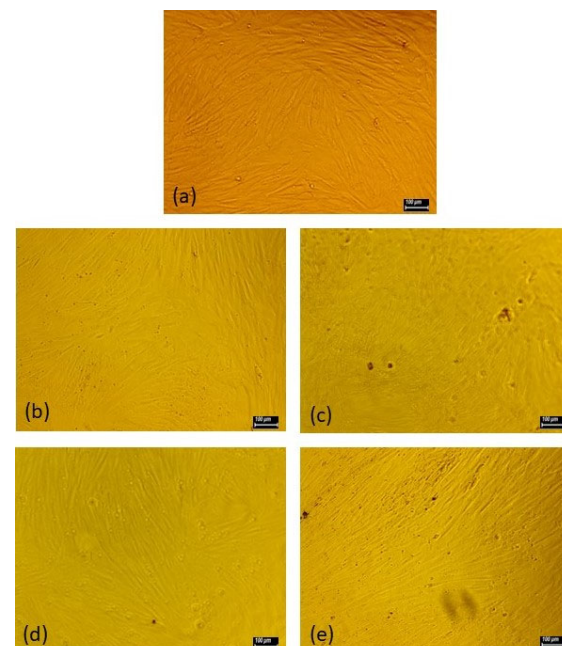


Figure 6. Rabbit MSC morphology after 3-day incubation in 7-day scaffolds extracts. (a) Control; (b) CH-A—chitosan scaffolds after alkaline treatment; (c) CH-HA-A—chitosan scaffolds with HA after alkaline treatment; (d) CH-T—chitosan scaffolds after temperature treatment; (e) CH-HA-T—chitosan scaffolds with HA after temperature treatment. Scale bar corresponding to 100 μm .

It should be noted that the morphology of cells cultured in the conditioned medium after incubation with the composite scaffolds treated with the HA particles that were converted to an insoluble form during alkaline treatment differed from the morphology of the cells in the control sample. Despite the almost complete confluence of the monolayer after 3 days of cultivation, the MSCs retained their round shape (Figure 6). On the remaining

three samples, the MSCs also formed a monolayer and the cells had an elongated fusiform shape, characteristic of MSC-like cells (Figure 6b,d,e) [51]. The morphology of the cells cultured on these three samples did not differ from that of the control sample, which was cultured in the standard nutrient medium. At the same time, the precipitation of the chitosan scaffolds without HA did not lead to any changes in the cell morphology.

To study the toxicity of the scaffolds, they were incubated in the nutrient medium, and cell viability was measured using an MTT assay. Figure 7 shows that the extracts obtained after incubating the chitosan scaffolds in the nutrient medium reduced the number of viable mesenchymal stem cells (MSCs). A slight decrease in viable cell number was observed in all samples, except for those incubated with composite scaffolds that had been treated with an alkaline solution. After one day of incubation, the alkaline substance adsorbed onto HA was extracted into the nutrient medium, leading to a 40% reduction in cell viability. It is worth noting that this alkaline extraction continued for the next seven days, and the viability of the cells cultured in the nutrient medium extract from the composite scaffolds coated with sodium hydroxide remained at a fairly low level compared to that of the control group.

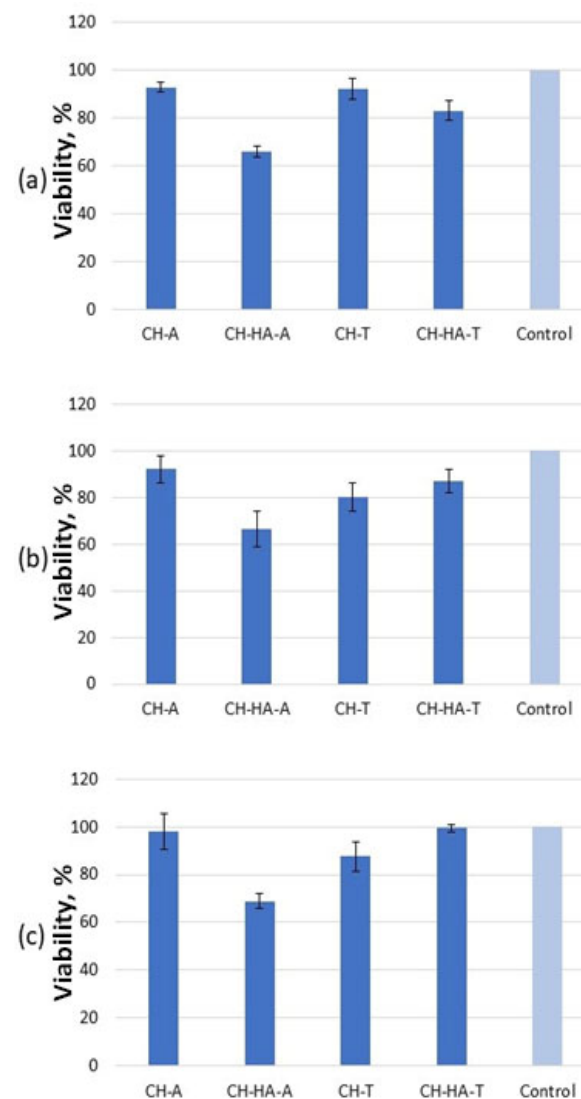


Figure 7. Viability of MSCs after 3 days of incubation: (a) 1-day extraction; (b) 3-day extraction; (c) 7-day extraction. CH-A—chitosan scaffolds after alkaline treatment; CH-HA-A—chitosan scaffolds with HA after alkaline treatment; CH-T—chitosan scaffolds after temperature treatment; CH-HA-T—chitosan scaffolds with HA after temperature treatment.

The presence of the HA particles in the thermo-modified composite scaffold caused a slight decrease in the viability of the cultured cells after 1 day (Figure 1A) of extraction. However, in the samples where the extraction was carried out for 3 days or more (Figure 7b,c), the number of viable MSCs after cultivation with extracts after incubation with CH-HA-T scaffolds was more comparable to that of viable MSCs after cultivation with extracts after incubation with CH-HA-A.

While heat treatment can indeed be considered as an alternative, it is not a complete replacement for alkali treatment. Alkali treatment has been well-established as a method that converts chitosan into insoluble form with a high degree of success. However, heat treatment only partially converts chitosan, which is why we observe differences in the mechanical properties of heat-treated and alkali-treated composite scaffolds. Heat-treated scaffolds exhibit greater elasticity compared to those treated with alkali, but they also absorb water and swell due to the incomplete conversion of chitosan. Therefore, while heat treatment can be a viable alternative for certain applications, it should be carefully considered in light of the specific requirements of the project.

4. Conclusions

The study shows that the use of sodium hydroxide to convert chitosan from its salt form, which is soluble in water, is only advisable for the formation of monocomponent chitosan scaffolds. The addition of the HA particles to the scaffold not only changes the mechanical properties of the composite scaffold but also requires additional conditions for converting the salt form of chitosan into an insoluble form. Of course, thermal modification is not the most effective method for obtaining the composite matrices, since it is impossible to fully convert the salt form of chitosan into insoluble chitosan, which is confirmed by our data and the literature. It should be noted that thermomodified matrices exhibit a decrease in mechanical properties compared to those treated with an alkaline solution. However, a significant reduction in the toxicity of the composite scaffolds obtained by thermal modification indicates the effectiveness of using this method of chitosan treatment for tissue engineering applications.

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Data Availability Statement: Data available upon request.

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