https://doi.org/10.15255/KUI.2022.018
KUI-59/2022
Original scientific paper
Received March 14, 2022
Accepted June 14, 2022

Boric Acid Modified Chitosan Scaffolds Chemically Crosslinked by Genipin

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Abstract

Chitosan scaffolds are an effective biologically active material with versatile application in chemistry and medicine. Chitosan is a linear polysaccharide, a derivative of chitin, with great biocompatibility due to the possession of functional groups such as −OH and $-NH_2$, which allow for biodegradability and antibacterial function. Chitosan has a polycation nature allowing complex formation with metal ions and many biomolecules such as DNA, proteins and lipids, while its specific structure and functional groups are responsible for antibacterial, hemostatic, and analgesic properties. To improve its angiogenic and antimicrobial potential, chitosan can be modified by boron (borate ions). The aim of this work was to prepare boric acid modified chitosan scaffolds, using boric acid as a boron precursor, as potential bioactive scaffolds for tissue regeneration. Borate ions tend to form complexes with hydroxyl groups, however, such physical interactions between boron and chitosan functional groups result in poor encapsulation efficiency. To ensure higher boron incorporation, chitosan scaffolds were cross-linked by genipin, a cross linker with lower cytotoxicity in contrast to glutaraldehyde commonly used to prepare stable chitosan-based materials. The degree of deacetylation (*DD*) and concentration of chitosan solution as well as the concentration of a solvent are important parameters that affect the crosslinking process. Moreover, the addition of boric acid could interfere with the crosslinking process by occupying chitosan functional groups. Here, chitosan scaffolds were modified with different concentrations of boric acid, while the concentrations of chitosan solution (1.2 w/v), genipin (2 % w/w), and acetic acid (0.5 % v/v) were kept constant. Obtained scaffolds were characterised by scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), differential scanning calorimetry (DSC), Fourier transformation infrared spectroscopy (FTIR), while cytotoxicity was evaluated as a function of materials concentration and exposure time. The results indicated successful incorporation of boron into crosslinked chitosan scaffolds with highly porous structure and low cytotoxicity.

Keywords

Chitosan, boron, scaffolds, genipin, cytotoxicity

1 Introduction

Natural human bone is a metabolically active tissue that undergoes continuous process of formation, resorption and remodelling. When it comes to small defects, bone possesses the self-healing ability.[1] However, larger defects originating from surgical resection require implants, *i.e.* scaffolds that serve as a temporary support for tissue repair. Development of potential substituent for bone tissue represents a great challenge due to high biological complexity of natural bone tissue and unique structure and properties. The properties of scaffolds are determined by their highly interconnected, three dimensional structure, tailored surface characteristics and mechanical properties.[2,3] The scaffold acts as a template to direct cell growth and extracellular matrix formation, while its architecture defines the ultimate shape of the new bone. Furthermore, scaffolds have to allow diffusion of necessary cell nutrients and proper diffusion of metabolic waste from the cells. More importantly, applied scaffolds should promote angiogenesis, which is essential for restoring biological and mechanical functionality of damaged bone.[4] Still, the ability of scaffolds to induce vascularisation as an initial step in new tissue growth, represents a major challenge in bone tissue engineering. Sufficient vascularisation is necessary for the repair of large bone defects, since it provides oxygen and nutrients, as well as transports osteoprogenitor cells to facilitate bone regeneration.[5]

In recent years, a considerable amount of research is being done on the application of chitosan in biomedicine. In the pharmaceutical field, chitosan has been found as a favourable drug delivery system,[6] while simultaneously, numerous studies are focused on the synthesis of chitosan-based porous implants which are conductive to bone regeneration.[7] Moreover, the field of tissue regeneration draws a lot of attention for novel chitosan regenerative biomaterials with angiogenic potential, and ability to accelerate revascularisation of damaged or treated tissue.[8] Chitosan is one of the most important products of thermochemical deacetylation of chitin. It is a linear polysaccharide, insoluble at pH > 7, while its biological characteristics offer considerable biocompatibility, nontoxicity, biodegradability, hydrophilicity, and antibacterial properties.[9,10] It consists of hydroxyl (−OH) and amino ($-NH_2$) functional groups giving it a cationic polymer nature. Moreover, chitosan ex-

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The paper was presented at XIV Meeting of Young Chemical Engineers 2022

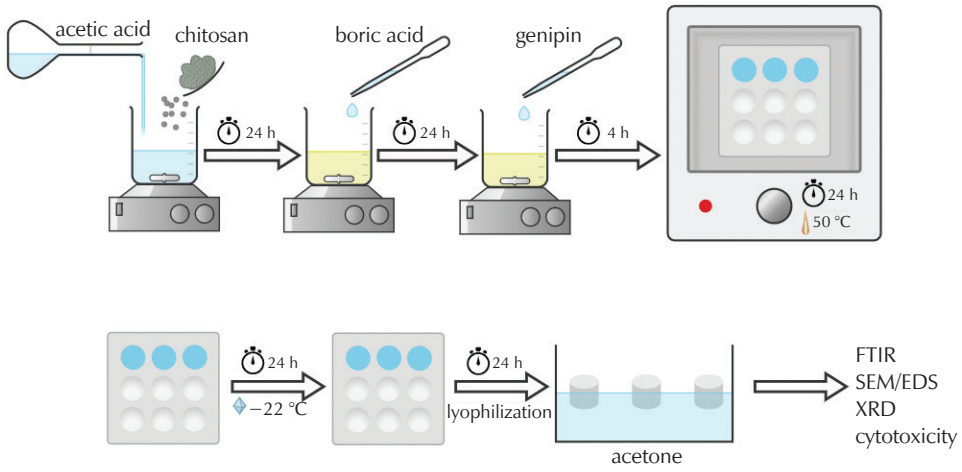

Fig. 1 – Schematic illustration of the material preparation
Slika 1 – Shematska ilustracija pripreme materijala

hibits a positive effect on intestinal flora balance, prevents growth of harmful bacteria, and can boost tissue regeneration due to its angiogenic properties.[11] Hydrogel scaffolds are amongst the most favourable forms of chitosan-based biomaterials. They are cost-effective and suitable due to their fluid absorption, retention, and drug carrying capacity.[12,13] Hydrogels also possess mechanical and structural properties comparable to the extracellular matrix (ECM) and allow for less disruptive implementation.[14] To enhance the wound healing process, chitosan is used in conjunction with other natural polymers such as gelatin, hyaluronic acid, collagen, cellulose or with inorganic components and metallic ions such as zinc, copper, and silver.[15,16]

Recently, boron is recognised as an element predominant for homeostasis of the human body. Previous works indicate that boron reacts with collagenase, elastase, and trypsin like enzymes in order to improve production of ECM.[17] Furthermore, there is growing interest in boron homeostasis, more precisely, in sodium boron cotransporter 1 (NaBC1), which controls boron homeostasis and takes part in myogenic differentiation.[18] Boron presence also enhances the availability of tumour necrosis factor – α (TNF-α) in fibroblasts, which in turn induces angiogenesis, augments gene expression responsible for inflammatory mediators, and represses the transcription of collagen gene.[19,20] Among precursors of boron, boric acid stands out as a long used medium for deep wound healing as it possesses antimicrobial activity.[21]

The aim of this study was to synthesise boric acid modified chitosan scaffolds using boric acid as a boron precursor. The successful encapsulation of boric acid was accomplished by chemical cross-linking using genipin. The presence of boric acid was indicated by Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction analysis, and energy dispersive X-ray spectroscopy (EDS), while highly porous structure was confirmed by SEM imaging. Moreover, the cytotoxic assay on human embryonic kidney (HEK 293) cell line indicated low cytotoxicity of boric acid modified chitosan scaffolds at lower material concentration.

2 Experimental part

2.1 Materials

Chitosan (CHT) with a degree of deacetylation (*DD*) of 83 % and viscosity of 293 mPa was purchased from Heppe (Germany). Acetic acid (99.8 %) and boric acid (p.a.) were purchased from Lachner (Czech Republic), acetone (p.a.) was purchased from T.T.T (Croatia), genipin was purchased from Cayman Chemical Company (USA).

2.2 Preparation of boric acid modified chitosan scaffolds

Chitosan was dissolved in 0.5 % (v/v) acetic acid, and stirred for five hours to obtain 1.2 % (w/v) chitosan solution. Different aliquots of boric acid were added to the chitosan solution resulting in concentrations of 10, 20, 50, and 100 mmol dm^{-3} in final chitosan/boric acid solution. After 24 h of stirring, genipin was added as cross-linking agent (2 % w/w) and stirring continued for 4 h. After homogenisation, prepared solutions were cast into a 24-well plate, and heated at 50 °C overnight due for cross-linking reaction. Cross-linked hydrogels were then frozen at −22 °C for 24 h, and lyophilised at −100 °C under vacuum for 48 h using a Kambic LIO-5PLT freeze-dryer. The dried scaffolds were then extensively washed with acetone to remove unreacted genipin. Obtained boric acid modified chitosan scaffolds with concentration of boric acid 0–100 mmol dm^{-3} were denoted as BA10, BA20, BA50, BA100. Pure chitosan scaffolds denoted as CHT were used as a control in X-ray diffraction analysis. A schematic illustration of the material preparation is given in Fig. 1.

2.3 Characterisation of boric acid modified scaffolds

The identification of obtained scaffolds was carried out using PerkinElmer instrument Spectrum One FT-IR spectrometer at 20 °C, resolution of 2 cm^{-1} and in spectral range of 4000–650 cm^{-1} with 32 scans.

X-ray diffraction data of the obtained scaffolds was collected at room temperature on Bruker D8 Discover diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) equipped with a LYNXEYE XE-T detector, in Bragg–Brentano geometry. X-ray source was Cu tube with a wavelength of 1.54060 Å powered at 1600 W (40 kV and 40 mA) with Ni filter, 2.5° soller slit and fixed slit at 0.4 mm. In the front of detector slit opening were 6.5 mm and detector opening was 1.3°. Diffraction peaks were followed from 3° to 70° 2θ with air scatter at a fixed distance of 0.5 mm from the sample.

Thermal behaviour of prepared scaffolds was investigated by DSC analysis on DSC 3500 Sirius Differential Scanning Calorimeter. The analysis was performed in an alumina crucible in the temperature range from 20 °C to 500 °C with a heating rate of 20 °C min^{-1} in a nitrogen atmosphere with a flow rate of 70 ml min^{-1}.

The morphology and elemental composition of the obtained boric acid modified scaffolds were analysed using scanning electron microscope TESCAN Vega3SEM Easyprobe with the electron beam energy of 10 keV equipped with EDS spectrometer Oxford INCA X-sight. Prior to imaging, the samples were sputtered with gold/palladium plasma for 120 s using Quorum SC 7620 sputter coater.

2.4 Cytotoxicity evaluation

The cytotoxic effects of boric acid modified scaffolds were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on human embryonic kidney (HEK 293) cell line. MTT assay is a standard colorimetric assay that measures the metabolic activity of cells.

HEK293 cells were cultured in Dulbecco's modified Eagle medium with 4500 mg l^{-1} glucose (DMEM-high glucose; Capricorn Scientific) supplemented with 10 % foetal bovine serum (FBS; Sigma-Aldrich), and 1 % penicillin/streptomycin (Sigma-Aldrich). After reaching 80 % confluence, the cells were seeded into a 96-well plate (Sarsted) at a density of $5 \cdot 10^4$ cells/200 µl of the medium, and allowed to adhere overnight in a humidified incubator with 5 % CO_2 at 37 °C.

Meanwhile, the samples of boric acid modified scaffolds (10 mg ml^{-1}, 1 mg ml^{-1}, and 0.5 mg ml^{-1}) were incubated in 3.5 ml volume of Dulbecco's modified Eagle medium for 24 h at 37 °C, 5 % CO_2. After incubation, the samples were vortexed then centrifuged (400 g/5 min). The cell culture medium was replaced by the supernatant from samples extract in 200 µl/well and incubated at 37 °C, 5 % CO_2 for 24, 48, and 72 h. Each sample was tested in triplicate.

Following the different incubation periods, 24, 48, and 72 h, the medium was removed and the cells were treated with 40 µl/well of MTT solubilised in the cell medium at a concentration of 0.5 mg ml^{-1}. After 3.5 h of incubation, 170 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each well to dissolve formed crystals (15 min). After 15 min of dissolving, absorbance was measured at 560 nm using the microplate reader (Glomax-Multi, Promega). Cell viability was calculated as a percentage of untreated control after reduction of blank absorbance on all samples.

3 Results and discussion

3.1 FTIR analysis

FTIR spectroscopy was used to identify possible interactions between chitosan and boric acid in boric acid modified scaffolds. FTIR spectra of all prepared scaffolds are given in Fig. 2. Genipin-crosslinked chitosan scaffold without boric acid (BA0) shows characteristic absorption bands: the region between 3350 cm^{-1} and 3180 cm^{-1} corresponds to the overlap of stretching vibration bands of −OH and $-NH_2$ groups, the absorption band at 2875 cm^{-1} is associated with symmetric stretching of $-CH_2$, the two absorption bands at 1650 cm^{-1} and 1555 cm^{-1} can be attributed to amide I and amide II stretching, the absorption bands at 1420 cm^{-1} and 1370 cm^{-1} can be assigned to C−N and $-CH_3$, and the absorption bands in the range

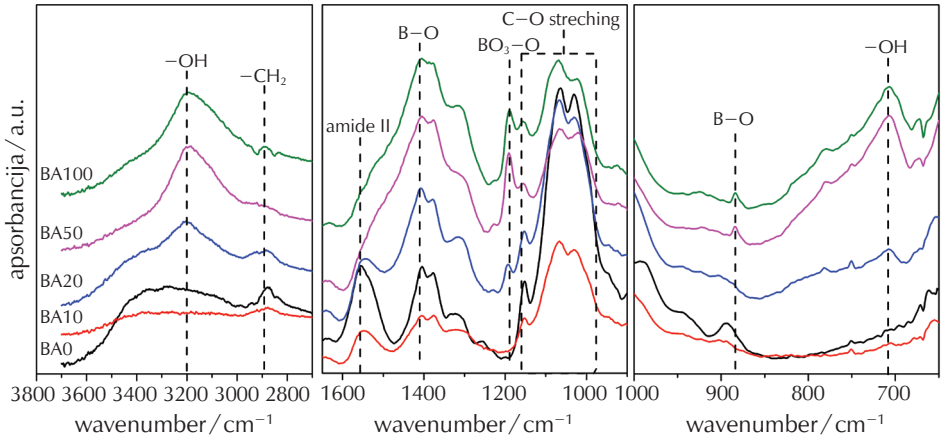

Fig. 2 – FTIR spectra of boric acid modified chitosan scaffolds
Slika 2 – FTIR spektri bornom kiselinom modificiranih kitozanskih nosača

of 1170–1000 cm^{-1} can be attributed to C−O stretching. The addition of boric acid brought changes in the intensity and shape of absorption bands at 1406 cm^{-1} causing the band broadening due to the absorption band assigned to asymmetric B−O stretching, which is more visible in the samples with higher BA concentrations (BA50 and BA100). Furthermore, the presence of boric acid in boric acid modified scaffolds is indicated by the absorption bands at 3210 cm^{-1}, 1189 cm^{-1}, 883 cm^{-1}, 708 cm^{-1} which are attributed to O−H stretching, BO_3−O stretching, B−O stretching, and −OH stretching of trigonal boric acid.[22,8,23,24] The possible interactions between chitosan functional groups and borate ions have been discussed in previous works. *Uddin et al.*[25] hypothesised on the intermolecular hydrogen bonding chitosan–boric acid, while *Saita et al.*[26] suggested a chitosan–borate complex, which may result in chitosan crosslinking. According to our FTIR spectra, a slight shift to lower wavenumbers of absorption band corresponding to amide II was observed in boric acid modified scaffolds with lower BA concentrations: from 1555 cm^{-1} to 1551 cm^{-1} in BA10 scaffold, and from 1555 cm^{-1} to 1549 cm^{-1} in BA20 scaffold, which could indicate possible physical interactions between borate ions and amide group of chitosan. The addition of a larger amount of boric acid to chitosan resulted in band overlap, which made it difficult to analyse the shift of the amide II band for the rest of the modified scaffolds.

3.2 X-ray diffraction

X-ray patterns of boric acid modified chitosan scaffolds, chitosan scaffold and boric acid powder are shown in Fig. 3. According to the pure chitosan scaffold, typical broad diffraction maximum at $2q \approx 20°$ was detected. Adding boric acid at the highest concentration (BA100) resulted in detection of two clearly observable diffraction maxima at $2q = 15°$ and 28°, which correspond to strongest diffraction maxima of boric acid. Another diffraction maximum at $2q \approx 12.5°$ was detected in BA100, which could correspond to semi-crystalline chitosan acetate, a crystal polymorph of chitosan salt type II with a helical asymmetric unit.[27,28] The major problem during synthesis of chitosan–boric acid scaffold is the efficiency of encapsulation of boric acid. Chitosan-based scaffolds can be prepared by physical or chemical cross-linking, where physical cross-link is based on the precipitation of chitosan solution using hydroxide solution (usually sodium hydroxide). This process is allowed due to chitosan solubility which is strongly affected by primary amine groups.[29] However, such preparation protocol includes neutralisation and subsequent washing step, resulting in the removal of boric acid from the material. In this work, boric acid was retained by chemical cross-link using genipin, an aglycone of geniposide, which can interact with the primary amine functional group of chitosan that causes cross-linking.[30,31] Still, even with the chemical cross-link of chitosan/BA scaffolds, a certain loss of boric acid from the scaffolds (data not shown) during final washing step with acetone was observed. It can be assumed that a certain amount of boric acid was removed along with unreacted genipin. Further analyses are needed to determine the concentration of boric acid in final scaffolds. The X-ray patterns of the scaffolds containing lower concentrations of boric acid (BA10, BA20, BA50) showed no diffraction maxima characteristic for boric acid, the presence of which was confirmed by FTIR analysis. This could be due to the lower detection limit of X-ray diffraction analysis.

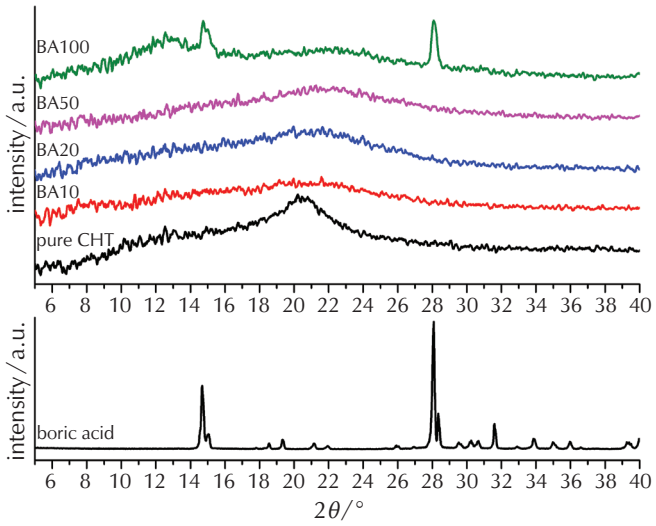

Fig. 3 – X-ray diffraction patterns of investigated samples
Slika 3 – Difraktogrami istraživanih sustava

3.3 DSC analysis

Fig. 4 depicts the DSC curves of BA0 and BA100 samples. BA0 sample shows an endothermic peak at 81 °C, which could be assigned to evaporation of water linked through hydrogen bonds due to strong affinity of polysaccharides for water,[32] and an exothermic peak at 290 °C corresponding to chitosan degradation, which was previously reported by *Guinesi et al.*[33] The addition of boric acid in BA100 sample caused changes in thermal behaviour: besides an endothermic peak at 80 °C, which could be assigned to water

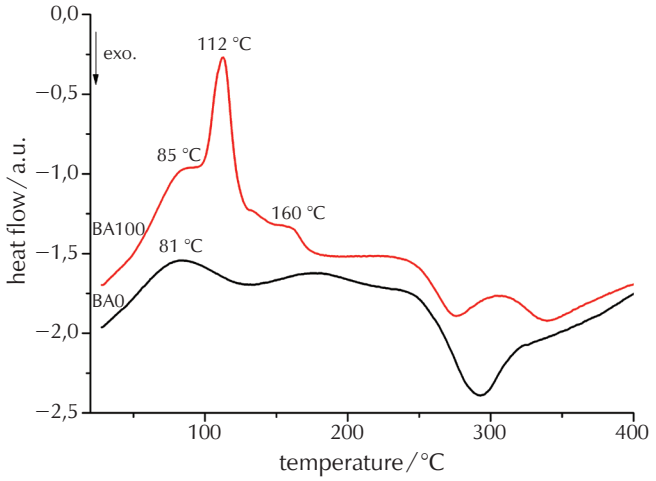

Fig. 4 – DSC curve of BA0 and BA100 samples
Slika 4 – DSC krivulja BA0 i BA100 uzoraka

loss, two new endothermic peaks at 112 and 160 °C were detected. Those effects could be due to decomposition of boric acid. Two mechanisms of boric acid decomposition are proposed: two-step and three step decomposition with a temperature range of 110 to 140 °C at different heating rates.[34] Furthermore, an exothermic peak in BA100 sample has been shifted to a lower temperature of 275 °C, which could be due to the introduction of boric acid. Additional endothermic peak at 341 °C could be assigned to a multi-step degradation of chitosan-based matrix caused by boric acid presence. However, additional thermal analyses are needed to clarify this thermal behaviour.

3.4 Scaffold microstructure and elemental analysis

The microstructure of boric acid modified scaffolds was imaged by SEM. The micrographs of scaffolds surface (Fig. 5) showed a highly porous structure obtained due to ice nucleation during the freezing process while simultaneously containing favourable interconnection of pores, which has been previously reported.[35,36] Obtained porous structure is an important characteristic of scaffolds applied in tissue engineering, since it allows non-hindered diffusion of nutrients, oxygen, metabolic waste, biomolecules for cell proliferation and differentiation, and easy cell migration.[37] The minimum requirement for pore size is considered to be ~100 μm due to cell size, migration, and nutrient and waste transport.[29] Visual assessment of the pore size of BA0 scaffold indicated size up to 200 mm, while the addition of boric acid had not altered the scaffolds microstructure significantly. Obtained porous structure of boric acid modified scaffolds could be suitable for future cell culture and tissue growth.

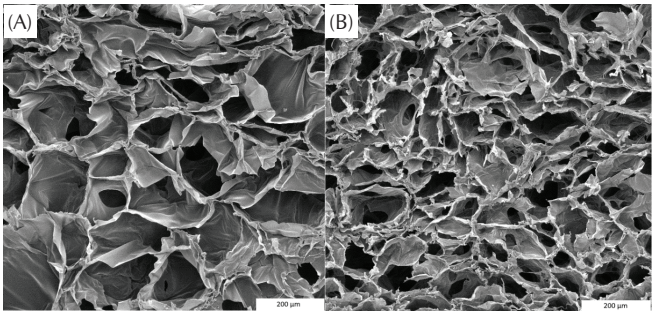

Fig. 5 – SEM micrographs of (A) BA0 and (B) BA100 scaffolds
Slika 5 – SEM mikrografije (A) BA0 i (B) BA100 nosača

The atomic composition of boric acid modified chitosan scaffolds was analysed using energy-dispersive X-ray analysis. As seen in Fig. 6A, EDS spectra of BA0 confirmed the presence of carbon and oxygen originated from chitosan. At the highest concentration of boric acid, BA100 scaffolds (Fig. 6B) showed the presence of boron at 0.18 keV. EDS analysis of boric acid modified scaffolds with lower concentration of BA were inconclusive due to the detection limit.

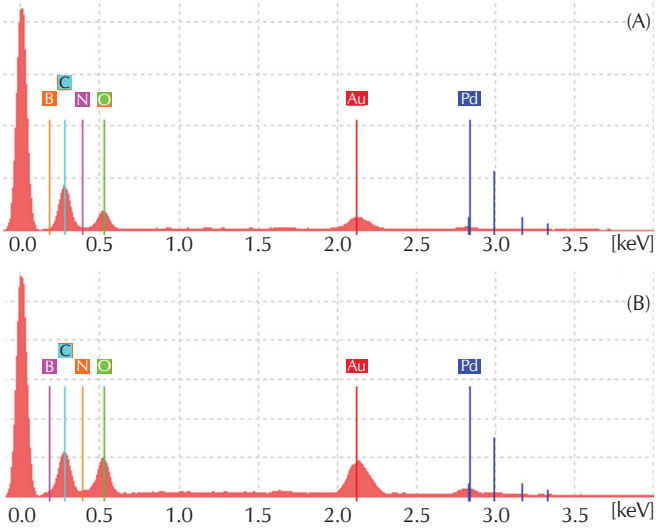

Fig. 6 – EDS analysis of (A) BA0 and (B) BA100 scaffolds
Slika 6 – EDS analiza (A) BA0 i (B) BA100 nosača

3.5 Cytotoxicity evaluation

The cytotoxicity assay is one of the basic tests to evaluate toxicity of materials potentially used in tissue engineering. In this work, the cytotoxicity test was performed on HEK293 cells cultured in material extract for 1, 2, and 3 days. Three different concentrations of boric acid modified scaffolds (10 mg ml^{-1}, 1 mg ml^{-1} and 0.5 mg ml^{-1}) were tested on cell viability (Fig. 7). Eighty percent of cell viability was considered as the materials cytotoxic limit. The cytotoxicity of boric acid modified scaffolds showed concentration-dependence during 3 days of cell culture. The cells cultured with a lower concentration of boric acid modified scaffolds (0.5 and 1 mg ml^{-1}) showed high viability, indicating non-cytotoxicity of the materials. The cytotoxicity of all prepared materials was observed at the highest concentration (10 mg ml^{-1}) with an almost linear increase in cytotoxicity by addition of boric acid. It was previously found that boric acid increases the production of RNA and translation of proteins that encode growth factors involved in angiogenesis and wound repair (such as VEGF and TGFb).[38] Moreover, it can trigger nitrogen-activated protein kinase signalling pathway, which increases cell proliferation at lower concentrations and inhibits it at higher boron concentration.[39] Additional impact on cell viability has the boron release profile. *Balasubramanian et al.*[40] reported the importance of the dissolution rate of boron-doped bioactive glasses on cell viability of ST2 cells. They observed poor cytocompatibility of bioactive glasses with high dissolution rate caused by high rate of boron release. In this work, lower cell viability during first and second days of culture was observed for higher concentrations of boric acid modified scaffolds, while the third day of culture indicated better cell viability when material concentrations were 0.5 and 1 mg ml^{-1}.

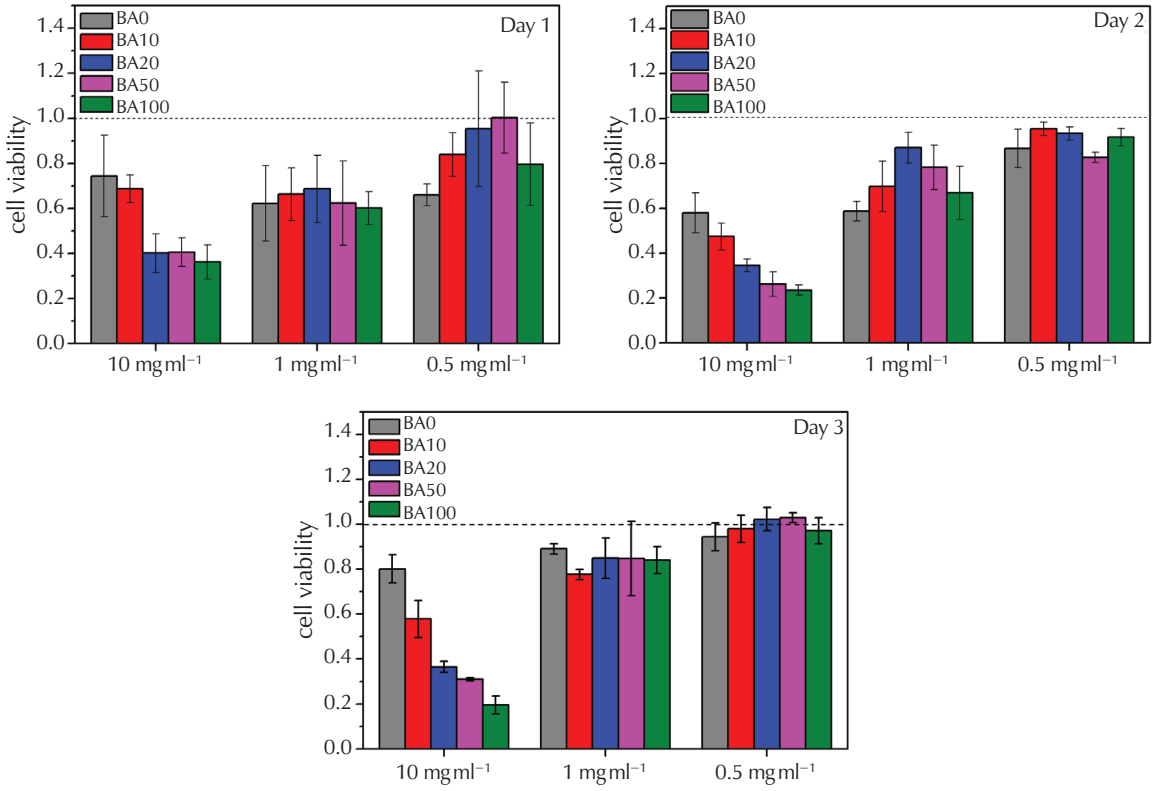

Fig. 7 – MTT assay of HEK293 human embryonic kidney cell line cultured in extracts of boric acid modified chitosan scaffolds for 1, 2, and 3 days

Slika 7 – MTT test vijabilnosti HEK293 stanica uzgojenih ekstraktom bornom kiselinom modificiranih kitozanskih nosača tijekom 1, 2 i 3 dana

4 Conclusion

Boric acid modified biomaterials show great potential in angiogenesis, which is a key process for tissue repair. In this work, boric acid modified chitosan scaffolds with different concentrations of boric acid, as a precursor for boron, were synthesised. Successful encapsulation of boric acid was obtained by chemical cross-linking with genipin while maintaining the highly porous structure necessary for the tissue engineering application. More importantly, the prepared scaffolds were non-cytotoxic to HEK293 cells when in contact with lower material concentrations. It can be concluded that the obtained boric acid modified scaffolds show good potential for further biological evaluation on mesenchymal stem cells.

ACKNOWLEDGEMENTS

This work has been supported by the Croatian Science Foundation under the project UIP-2020-02-6201. T.K. acknowledges CeNIKS project financed from the European Regional Development Fund, OP "Competitiveness and Cohesion" 2014–2020.

List of abbreviations and symbols
Popis kratica i simbola

CHT – chitosan
– kitozan

FTIR – Fourier-transform infrared spectroscopy
– infracrvena spektroskopija s Fourierovom transformacijom

DSC – differential scanning calorimetry
– diferencijalna pretražna kalorimetrija

EDS – energy dispersive X-ray spectroscopy
– energetski disperzivna rendgenska spektroskopija

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
– 3-(4, 5-dimetiltiazol-2-il)-2, 5-difeniltetrazolium bromid

DMEM – Dulbecco's modified Eagle medium
– Eagleov medij koji je modificirao Dulbecco

DMSO – dimethyl sulfoxide
– dimetil sulfoksid

SEM – scanning electron microscopy
– pretražna elektronska mikroskopija

HEK – human embryonic kidney 293 cells
– embrionalne stanice bubrega čovjeka

References
Literatura

1. *R. Dimitriou, E. Jones, D. McGonagle, P. V Giannoudis*, Bone regeneration: current concepts and future directions, BMC Med. **9** (2011) 66, doi: https://doi.org/10.1186/1741-7015-9-66.
2. *D. W. Hutmacher*, Scaffolds in tissue engineering bone and cartilage, Biomaterials **21** (2000) 2529–2543, doi: https://doi.org/10.1016/S0142-9612(00)00121-6.
3. *A. R. Boccaccini, I. Notingher, V. Maquet, R. Jérôme*, Bioresorbable and bioactive composite materials based on polylactide foams filled with and coated by Bioglass particles for tissue engineering applications, J. Mater. Sci. Mater. Med. **14** (2003) 443–450, doi: https://doi.org/10.1023/A:1023266902662.
4. *M. W. Laschke, M. D. Menger*, Vascularization in tissue engineering: Angiogenesis *versus* inosculation, Eur. Surg. Res. **48** (2012) 85–92, doi: https://doi.org/10.1159/000336876.
5. *W. Yu, T.-W. Sun, Z. Ding, C. Qi, H. Zhao, F. Chen, Z. Shi, Y.-J. Zhu, D. Chen, Y. He*, Copper-doped mesoporous hydroxyapatite microspheres synthesized by a microwave-hydrothermal method using creatine phosphate as an organic phosphorus source: application in drug delivery and enhanced bone regeneration, J. Mater. Chem. B **5** (2017) 1039–1052, doi: https://doi.org/10.1039/C6TB02747D.
6. *T. Kean, M. Thanou*, Biodegradation, biodistribution and toxicity of chitosan, Adv. Drug Deliv. Rev. **62** (2010) 3–11, doi: https://doi.org/10.1016/j.addr.2009.09.004.
7. *R. C. Goy, D. de Britto, O. B. G. Assis*, A review of the antimicrobial activity of chitosan, Polímeros **19** (2009) 241–247, doi: https://doi.org/10.1590/S0104-14282009000300013.
8. *S. Ahtzaz, T. Sher Waris, L. Shahzadi, A. Anwar Chaudhry, I. Ur Rehman, M. Yar*, Boron for tissue regeneration-it's loading into chitosan/collagen hydrogels and testing on chorioallantoic membrane to study the effect on angiogenesis, Int. J. Polym. Mater. Polym. Biomater. **69** (2020) 525–534, doi: https://doi.org/10.1080/00914037.2019.1581202.
9. *K. Prusty, S. K. Swain*, Chitosan-Based Nanobiocomposites for Wound-Healing Applications, in S. K. Swain, M. Jawaid (Eds.), Nanostructured Polymer Composites for Biomedical Applications, Elsevier, Chennai, India, 2019, pp. 295–314.
10. *Y. Bing, L. XingYi, S. Shuai, K. XiangYe, G. Gang, H. MeiJuan, L. Feng, X. Z. YuQuan, Q. ZhiYong*, Preparation and characterization of a novel chitosan scaffold, Carbohydr. Polym. **80** (2010) 860–865, doi: https://doi.org/10.1016/j.carbpol.2009.12.044.
11. *R. A. A. Muzzarelli*, Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone, Carbohydr. Polym. **76** (2009) 167–182, doi: https://doi.org/10.1016/j.carbpol.2008.11.002.
12. *L. Christenson, A. G. Mikos, D. F. Gibbons, G. L. Picciolo*, Biomaterials for Tissue Engineering: Summary, Tissue Eng. **3** (1997) 71–76, doi: https://doi.org/10.1089/ten.1997.3.71.
13. *A. Aguirre, A. González, M. Navarro, Ó. Castaño, J. Planell, E. Engel*, Control of microenvironmental cues with a smart biomaterial composite promotes endothelial progenitor cell angiogenesis, Eur. Cells Mater. **24** (2012) 90–106, doi: https://doi.org/10.22203/eCM.v024a07.
14. *K. Y. Lee, D. J. Mooney*, Hydrogels for Tissue Engineering, Chem. Rev. **101** (2001) 1869–1880, doi: https://doi.org/10.3390/polym3041776.
15. *H. N. Pei, X. G. Chen, Y. Li, H. Y. Zhou*, Characterization and ornidazole release in vitro of a novel composite film prepared with chitosan/poly(vinyl alcohol)/alginate, J. Biomed. Mater. Res. Part A **85A** (2008) 566–572, doi: https://doi.org/10.1002/jbm.a.31223.
16. *L. Yangshuo, K. Hyung-Il*, Characterization and antibacterial properties of genipin-crosslinked chitosan/poly(ethylene glycol)/ZnO/Ag nanocomposites, Carbohydr. Polym. **89** (2012) 111–116, doi: https://doi.org/10.1016/j.carbpol.2012.02.058.
17. *M. Benderdour, T. Van Bui, K. Hess, A. Dicko, F. Belleville, B. Dousset*, Effects of boron derivatives on extracellular matrix formation, J. Trace Elem. Med. Biol. **14** (2000) 168–173, doi: https://doi.org/10.1016/S0946-672X(00)80006-1.
18. *P. Rico, A. Rodrigo-Navarro, L. S. Pérez, M. Salmeron-Sanchez*, Boron induces osteogenesis by stimulating NaBC1 in cooperation with BMPR1A, bioRxiv **1** (2020) 995001, doi: https://doi.org/10.1101/2020.03.17.995001.
19. *M. Benderdour, K. Hess, M. Dzondo-Gadet, P. Nabet, F. Belleville, B. Dousset*, Boron Modulates Extracellular Matrix and TNFα Synthesis in Human Fibroblasts, Biochem. Biophys. Res. Commun. **246** (1998) 746–751, doi: https://doi.org/10.1006/bbrc.1998.8688
20. *A. Hatamochi, K. Mori, H. Ueki*, Role of cytokines in controlling connective tissue gene expression, Arch. Dermatol. Res. **287** (1994) 115–121, doi: https://doi.org/10.1007/BF00370729.
21. *N. Roy, N. Saha, T. Kitano, P. Saha*, Development and Characterization of Novel Medicated Hydrogels for Wound Dressing, Soft Mater. **8** (2010) 130–148, doi: https://doi.org/10.1080/15394451003756282.
22. *D. E. Bethell, N. Sheppard*, The infra-red spectrum and structure of boric acid, Trans. Faraday Soc. **51** (1955) 9–15, doi: https://doi.org/10.1039/TF9555100009.
23. *A. Harabor, P. Rotaru, R. I. Scorei, N. A. Harabor*, Non-conventional hexagonal structure for boric acid, J. Therm. Anal. Calorim. **118** (2014) 1375–1384, doi: https://doi.org/10.1007/s10973-014-4169-5.
24. *D. Peak, G. W. Luther, D. L. Sparks*, ATR-FTIR spectroscopic studies of boric acid adsorption on hydrous ferric oxide, Geochim. Cosmochim. Acta **67** (2003) 2551–2560, doi: https://doi.org/10.1016/S0016-7037(03)00096-6.
25. *K. M. A. Uddin, M. Ago, O. J. Rojas*, Hybrid films of chitosan, cellulose nanofibrils and boric acid: Flame retardancy, optical and thermo-mechanical properties, Carbohydr. Polym. **177** (2017) 13–21, doi: https://doi.org/10.1016/j.carbpol.2017.08.116.
26. *K. Saita, S. Nagaoka, T. Shirosaki, M. Horikawa, S. Matsuda, H. Ihara*, Preparation and characterization of dispersible chitosan particles with borate crosslinking and their antimicrobial and antifungal activity, Carbohydr. Res. **349** (2012) 52–58, doi: https://doi.org/10.1016/j.carres.2011.12.017.
27. *G. C. Ritthidej, T. Phaechamud, T. Koizumi*, Moist heat treatment on physicochemical change of chitosan salt films, Int. J. Pharm. **232** (2002) 11–22, doi: https://doi.org/10.1016/S0378-5173(01)00894-8.
28. *D. Magnin, S. Dumitriu,* Interactions between polysaccharides and polypeptides, in Severian Dimitru (Ed.) Polysaccharides: Structural diversity and functional versatility, CRC press, New York, 2004, pp. 305–356.
29. *K. Harish Prashanth*, Solid state structure of chitosan prepared under different N-deacetylating conditions, Carbohydr. Polym. **50** (2002) 27–33, doi: https://doi.org/10.1016/S0144-8617(01)00371-X.
30. *Y. Yu, S. Xu, S. Li, H. Pan*, Genipin-cross-linked hydrogels based on biomaterials for drug delivery: a review, Biomater. Sci. **9** (2021) 1583–1597, doi: https://doi.org/10.1039/D0BM01403F.
31. *S. Dimida, C. Demitri, V. M. De Benedictis, F. Scalera, F.*

Gervaso, A. Sannino, Genipin-cross-linked chitosan-based hydrogels: Reaction kinetics and structure-related characteristics, J. Appl. Polym. Sci. **132** (2015), doi: https://doi.org/10.1002/app.42256.

32. *J. Ostrowska-Czubenko, M. Gierszewska-Drużyńska*, Effect of ionic crosslinking on the water state in hydrogel chitosan membranes, Carbohydr. Polym. **77** (2009) 590–598, doi: https://doi.org/10.1016/j.carbpol.2009.01.036.
33. *L. S. Guinesi, É. T. G. Cavalheiro*, The use of DSC curves to determine the acetylation degree of chitin/chitosan samples, Thermochim. Acta **444** (2006) 128–133, doi: https://doi.org/10.1016/j.tca.2006.03.003.
34. *C. Huber, S. S. Jahromy, F. Birkelbach, J. Weber, C. Jordan, M. Schreiner, M. Harasek, F. Winter*, The multistep decomposition of boric acid, Energy Sci. Eng. **8** (2020) 1650–1666, doi: https://doi.org/10.1002/ese3.622.
35. *S. Takeshita, S. Zhao, W. J. Malfait, M. M. Koebel*, Chemistry of Chitosan Aerogels: Three-Dimensional Pore Control for Tailored Applications, Angew. Chemie Int. Ed. **60** (2021) 9828–9851, doi: https://doi.org/10.1002/anie.202003053.
36. *S. V. Madihally, H. W. T. Matthew*, Porous chitosan scaffolds for tissue engineering, Biomaterials **20** (1999) 1133–1142, doi: https://doi.org/10.1016/S0142-9612(99)00011-3.
37. *S. B. Qasim, S. Husain, Y. Huang, M. Pogorielov, V. Deineka, M. Lyndin, A. Rawlinson, I. U. Rehman*, *In-vitro* and *in-vivo* degradation studies of freeze gelated porous chitosan composite scaffolds for tissue engineering applications, Polym. Degrad. Stab. **136** (2017) 31–38, doi: https://doi.org/10.1016/j.polymdegradstab.2016.11.018.
38. *M. Dzondo-Gadet, R. Mayap-Nzietchueng, K. Hess, P. Nabet, F. Belleville, B. Dousset*, Action of Boron at the Molecular Level Effects on Transcription and Translation in an Acellular System, Biol. Trace Elem. Res. **85** (2002) 23–33, doi: https://doi.org/10.1385/BTER:85:1:23.
39. *M. Park, Q. Li, N. Shcheynikov, W. Zeng, S. Muallem*, NaBC1 Is a Ubiquitous Electrogenic Na^+-Coupled Borate Transporter Essential for Cellular Boron Homeostasis and Cell Growth and Proliferation, Mol. Cell **16** (2004) 331–341, doi: https://doi.org/10.1016/j.molcel.2004.09.030.
40. *P. Balasubramanian, L. Hupa, B. Jokic, R. Detsch, A. Grünewald, A. R. Boccaccini*, Angiogenic potential of boron-containing bioactive glasses: *in vitro* study, J. Mater. Sci. **52** (2017) 8785–8792, doi: https://doi.org/10.1007/s10853-016-0563-7.

SAŽETAK

Bornom kiselinom modificirani kitozanski nosači kemijski umreženi genipinom

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Kitozanski nosači su efektivni biološki aktivni materijali sa širokom primjenom u kemiji i medicini. Kitozan je linearni polisaharid, derivat hitina, koji posjeduje dobru biokompatibilnost koja se pripisuje prisustvu funkcionalnih skupina kao što su $-OH$ i $-NH_2$ koje pridonose biorazgradljivim i antibakterijskim svojstvima. U kiselim vodenim otopinama kitozan je polikationske strukturne prirode, koja ima veliku sposobnost stvaranja kompleksa s različitim metalnim ionima i važnim biomolekulama, kao što su DNA, proteini i lipidi. Jedinstvena struktura, kao i uvođenje specifičnih funkcionalnih skupina odgovorni su za antibakterijska svojstva, hemostatsku aktivnost i analgetska svojstva tog biopolimera. Za poboljšanje angiogenih i antimikrobnih svojstava, kitozan se može modificirati borom (boratnim ionima). Cilj ovog rada bio je pripremiti bornom kiselinom modificirane kitozanske nosače, upotrebljavajući bornu kiselinu kao prekursor bora, u svrhu priprave potencijalnih bioaktivnih okosnica za regeneraciju tkiva. Boratni ioni imaju sklonost stvaranja kompleksa s hidroksilnim skupinama, međutim, takve interakcije između funkcionalnih skupina kitozana i bora rezultiraju slabijom učinkovitosti njegove inkapsulacije. Da bi se osigurala bolja ugradnja bora, kitozanski nosači su umreženi genipinom, manje toksičnim umreživalom u odnosu na glutaraldehid koji se obično upotrebljava za pripremu stabilnih materijala čiji se sastav temelji na kitozanu. Stupanj deacetilacije (*DD*) i koncentracija otopine kitozana kao i koncentracija otapala važni su parametri koji utječu na proces umreživanja. Nadalje, dodatak borne kiseline mogao bi utjecati na proces umreživanja zauzimanjem funkcionalnih skupina kitozana. U ovom radu kitozanski nosači modificirani su bornom kiselinom različite koncentracije, dok su koncentracija otopine kitozana (1,2 w/v), koncentracija genipina (2 % w/w) i koncentracija octene kiseline (0,5 % v/v) bile konstantne. Dobiveni nosači okarakterizirani su pretražnom elektronskom mikroskopijom (SEM), energetski disperzivnom rendgenskom spektroskopijom (EDS), diferencijalnom pretražnom kalorimetrijom (DSC), infracrvenom spektroskopijom s Fourierovom transformacijom (FTIR), dok je citotoksičnost procijenjena kao funkcija koncentracije materijala i vremena izloženosti stanica materijalu. Rezultati su pokazali uspješnu ugradnju bora u umrežene kitozanske nosače, visoko poroznu strukturu i nisku citotoksičnost.

Ključne riječi
Kitozan, bor, okosnica, genipin, citotoksičnost

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Izvorni znanstveni rad
Prispjelo 14. ožujka 2022.
Prihvaćeno 14. lipnja 2022.