DOUBLE CROSSLINKING OF CHITOSAN/VANILLIN HYDROGELS AS A BASIS FOR MECHANICALLY STRONG GRADIENT SCAFFOLDS FOR TISSUE ENGINEERING

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Abstract

Polysaccharides, such as chitosan (CS), are widely used in many biomedical applications. However, they require crosslinking agents to achieve chemical stability and appropriate mechanical properties. In this work, chitosan-based hydrogels were crosslinked using vanillin and/or sodium tripolyphosphate, as chemical and physical crosslinking agents, respectively. Microstructural (digital microscope, SEM), structural (FTIR-ATR), mechanical (static compression test), and in vitro biological (chemical stability and swelling ratio in PBS, cytotoxicity) properties of the obtained materials were evaluated to assess materials potential as biomedical scaffolds. The optimal ratio of vanillin to chitosan (DD = 89%) to crosslink the polymer was found to be 1.2:1. Moreover, the double crosslinking with vanillin caused a two-time increase in the compression strength of the samples and led to the slower biodegradability. Cytotoxicity studies showed that the cells prefer double vanillin crosslinked hydrogels over those treated with TPP. Further studies, such as bioactivity are required to determine the specific functionality of the hydrogels and the specific tissue which may be treated with the tested materials. The optimal material was chosen to the next step of the study, which may be obtaining composite hydrogels with hydroxyapatite and/or graphene oxide to tailor or improve properties towards specific tissue regeneration.

Keywords: *hydrogel, chitosan, vanillin, tripolyphosphate, tissue engineering*

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Introduction

Polysaccharides, a large group of natural polymers such as chitosan (CS), hyaluronic acid (HA), alginates, gellan gum (GG) have been widely used to form hydrogels for tissue engineering applications. CS seems to be one of the most versatile among them [1]. Its polycationic nature and the presence of amino groups allow to form biocompatible scaffolds which are able to absorb large amounts of water and are prone to further modifications. Thus, CS-based systems mimicking many native tissues may be created for the regeneration of skin [2,3], bone [4,5], cartilage [6,7], nerves [8], or blood vessels [9].

There are many different ways to crosslink CS and thus form chemically stable and mechanically strong hydrogels, i.e. chemical, physical or metal-coordination-based crosslinking [10]. Chemically formed networks are created using dialdehydes, e.g. glutaraldehyde, yet most of them are considered cytotoxic. Genipin is a natural non-toxic agent which has also been widely used. As a crosslinking agent for CS, it improves the cell viability on hydrogels. However, the conditions of the crosslinking reaction influence the hydrogel structure significantly [11]. Another natural aldehyde, not only cyto-compatible but also much cheaper, is vanillin. There is evidence that vanillin improves the mechanical properties of the chitosan hydrogel [12]. Moreover, it is an antioxidant, which constitutes its significant advantage [13]. As a CS crosslinker, it is mainly used to obtain films [14], membranes [15] or drug microcarriers and microspheres [16,17]. However, the insufficient chemical stability of hydrogels crosslinked with vanillin requires additional modifications to prevent the CS matrix from being altered immediately after its generation.

The main disadvantage of covalent crosslinking is the chemical selectivity of the process which may lead to producing toxic side-products. Moreover, the limited control over the created microstructure makes this crosslinking method less desirable [18]. The click-chemistry reaction may be a more preferable solution but only a functionalized CS may be used to prepare hydrogels through this reaction [19]. The same limitation occurs when CS is crosslinked enzymatically, which makes these methods impractical [20].

Forming coordination complexes based on the reaction between amine or hydroxyl groups of CS and metal ions is yet another method to obtain the CS-based hydrogel. Although it is a quick process, the final crosslinked polymer is characterized by lower stability. In addition, this method is very sensitive to changes of the polysaccharide properties [21].

The physically crosslinked CS hydrogel is the result of forming CS polycations and electrostatic interactions with oppositely charged molecules. Ionic crosslinking may provide a decrease in the swelling ratio of the final hydrogel, dependant on the pH value [22]. One of the physical crosslinking agents used for CS is tripolyphosphate (TPP) [24]. This crosslinker is cyto-compatible and the final properties of CS-based hydrogel are easily tailorable, for example by different crosslinking degrees. On the other hand, the mechanical properties of physically crosslinked hydrogels are relatively low. In addition, thus obtained hydrogel exhibits a fast biodegradability [23-25] while, in bone tissue engineering scaffolds are required to be mechanically strong and stable under physiological conditions.

One of the solutions to improve the chemical stability and mechanical characteristic of the hydrogel is to apply the second crosslinking, which may increase both the hydrogel biodegradability and mechanical strength, due to the presence of additional bonds and a more tangled network. Additionally, using a natural crosslinking agent provides higher biocompatibility, therefore a combination of two crosslinking mechanisms may significantly improve the hydrogel properties [17].

This study involved a double-step crosslinking process for chitosan, based on chemical (vanillin) and/or physical (TPP) crosslinking. A natural polysaccharide, such as chitosan, and a natural crosslinking agent, such as vanillin were applied to improve the biocompatibility of the final hydrogel. Co-acting of the chemical and/or physical crosslinking reactions were studied to improve the mechanical properties and slow down biodegradation in the physiological environment.

Materials and Methods

Materials

Chitosan (CS; Mw = 100.000-300.000) was purchased from Acros Organics, USA. Avantor Performance Materials Poland S.A. reagents were used as follows: acetic acid (AAc; 99.9%), vanillin, sodium tripolyphosphate (TPP) and ethanol (EtOH; 96%). First, CS and vanillin powders were dissolved in 2% AAc and EtOH, respectively. As the first crosslinking step, the appropriate VAN solutions (1 ml) were added dropwise to the CS, mixed vigorously and homogenized by sonication (10 min). The final concentration of CS solution was controlled at 5% w/v of the final volume (20 ml). The mass ratios of CS:VAN and final concentrations of VAN are presented in TABLE 1. After mixing, the samples were left at room temperature and subsequently tested after 3, 4, 5 and 6 days of crosslinking. The 6x6 mm cubic samples of 1van, 1.2van, 1.4van were initially crosslinked with vanillin and maintained for 6 days. For the second crosslinking step they were cut and immersed in a vanillin solution (5% in EtOH, denoted as VAN_1, VAN_1.2, VAN_1.4) or a TPP solution (5% in distilled water, denoted as TPP_1, TPP_1.2, TPP_1.4) for 24 h to improve the crosslinking process. The proportion of hydrogel mass to crosslinking solution volume was 1g:10ml. Some of the samples were frozen at -80°C and freeze-dried for further analysis.

TABLE 1. The mass ratios of CS:VAN used in the study.

CS:VAN mass ratio	1:0.8	1:1	1:1.2	1:1.4	1:1.6
Sample description	0.8van	1van	1.2van	1.4van	1.6van
Final concentration of VAN [%]	4	5	6	7	8

Methods

Determination of a deacetylation degree (DD)

The deacetylation degree of chitosan used in this study was determined using the titration method [26]. Briefly, CS powder (0.2 g) dissolved in 0.1M HCl (20 ml) and deionized water (50 ml) was titrated with 0.1M NaOH. The pH changes dependent on titrant volume allowed to carry out the calculation using Formula 1:

$$DD = 2.03 \cdot \frac{V_2 - V_1}{m + 0.0042(V_2 - V_1)} \cdot 100\% \quad (1)$$

where: m – the exact mass of CS powder [g], V_1 and V_2 - volumes of 0.1M NaOH solution corresponding to the deflection points [ml], 2.03 – coefficient resulting from the molecular weight of chitin monomer unit, 0.0042 – coefficient connected with the difference between molecular weights of chitin and chitosan monomer units.

Chemical stability test

The biodegradation *in vitro* process was carried out in the PBS (Phosphate-Buffered Saline, pH = 7.4) solution at 37°C. The ratio of the sample mass to the PBS solution volume was 1g:100ml. The following time points were tested: 1, 2, 3, 4, 5, 6, 7, 14, 21 and 28 days. After the first 7 days of incubation, the PBS solution was substituted with the fresh one. At these intervals, the pH value was measured. After 28 days of incubation, the samples were washed with distilled water and weighed. The weight loss (WL) was calculated using Formula 2:

$$WL = \frac{M_0 - M_j}{M_0} \cdot 100\ [\%] \quad (2)$$

where: M_0 – the initial mass of the sample [g], M_j – the weight of the sample after 28 days of incubation [g]

Static compression test

The universal testing machine (Zwick 1435, Germany) was used to analyze the mechanical properties of the samples (static compression test). All the measurements were performed using wet cubic samples, at room temperature. The samples after 28 days of incubation were also tested (denoted as: VAN_1_PBS, VAN_1.2_PBS, VAN 1.4_PBS, TPP_1_PBS, TPP_1.2_PBS, TPP_1.4_PBS). The Young's modulus and compressive strength values (at 3 mm deformation) were evaluated; Young's modulus was determined based on a linear fragment of the stress-strain curve. The strain was evaluated at F=10N and the stress was measured at 3 mm of strain. The test speed was set on 2 mm/min.

Swelling ratio

The samples were weighed and immersed in the PBS solution at 37°C. The swelling ratios were assessed after 1, 2, 3, 4 and 5 hours of the incubation, and then daily up to the 5th day. At these points, the swollen samples were dried carefully with a tissue, then weighed and placed back in the PBS solutions. The calculations of swelling ratio (SR) were made using Formula 3:

$$SR = \frac{M_i - M_0}{M_0} \cdot 100\ [\%] \quad (3)$$

where: M_i – the weight of the swollen sample [g], M_0 – the initial mass of the sample [g]

Cytotoxicity test *in vitro*

The cytotoxicity studies were conducted using extracts in accordance with the ISO norm 10993-5 (2009). The materials were tested in contact with osteoblastogenic cells of the MG-63 line (European Cell Bank - European Collection of Cell Cultures, Sailsbury, UK). The cells were cultured in the EMEM medium (Eagle's Minimal Essential Medium, PAN BIOTECH, Germany) with the addition of 10% bovine serum (Fetal Bovine Serum, Biowest, France) and 1% antibiotics (penicillin/streptomycin, PAA, Austria), 0.1% amino acids and 0.1% pyruvate (PAA, Austria). The culture was carried out at 37°C, 5% CO_2 and under the increased humidity.

The extraction was performed based on the 100 mg:1 ml ratio of the tested material to the culture medium. The weighed freeze-dried samples were immersed in the culture medium (the exact masses and volumes are summarized in TABLE 2) and left for 24 h at 37°C. Then the extracts were sterilized via filtration using syringe filters (0.22 µm).

TABLE 2. Samples tested on extracts, the exact sample weights and medium volumes used for extraction.

Sample	Mass [mg]	Culture medium volume [ml]
VAN_1	423.23	4.230
VAN_1.2	426.60	4.265
VAN_1.4	423.05	4.230
TPP_1	433.07	4.330
TPP_1.2	416.32	4.165
TPP_1.4	416.27	4.165
Medium	-	4.000

The following dilutions of extracts were prepared in the study: 1 (undiluted), 1/2, 1/4, 1/8 and 1/16. The culture medium was used to dilute the extracts. As the reference sample served the culture medium maintained under the conditions identical to the ones of the tested samples (no dilutions were used for the medium).

The cells were placed in 96-well plates - 5,000 cells per well (100 µl medium). After 24 h of incubation, the supernatant medium was replaced with the appropriately diluted extracts (100 µl). After the next 24 h, cell metabolic activity and viability were tested using the AlamarBlue test and live/dead staining. For this purpose, the following chemicals and devices were used: In Vitro Toxicology Assay Kit, resazurin-based (Sigma Aldrich), calcein AM/propidium iodide (Sigma Aldrich), Zeiss Axiovert 40 fluorescence microscope (Carl Zeiss, Germany) and BMG Labtech spectrofluorimeter, FluoStar Omega.

The calculations of resazurin reduction (RR) were made by Formula 4:

$$RR\ [\%] = \frac{F_{sample} - F_{0\%\ red}}{F_{100\%\ red} - F_{0\%\ red}} \cdot 100\% \qquad (4)$$

where: F_{sample} - fluorescence of sample from culture well, $F_{0\%\ red}$ - fluorescence of culture medium with the addition of AlamarBlue reagent without cells, $F_{100\%\ red}$ - fluorescence of culture medium with the addition of AlamarBlue reagent reduced of 100% by autoclaving (15 min, 121°C).

The statistical analysis was performed using ANOVA (One Way Analysis of Variance) followed by Tukey post-hoc test. The significance levels were set at $p<0.05$ and $p<0.001$. The normality and equal variances were tested with the Shapiro-Wilk and Leuven test, respectively, at $p<0.05$.

Results and Discussion

The charts presenting the results after the CS powder titration are shown in FIG. 1. Based on the pH dependence on the NaOH volume added to the CS solution, the deacetylation degree of chitosan used was determined. After the calculation using Formula 1 the DD was found to be 89%. This parameter determined the CS properties, such as a susceptibility to modifications, the availability of the functional amino groups and solubility. It also possibly changed the crosslinking degree, thus influencing the mechanical properties or chemical stability. It is worth noting that the higher DD is desirable regarding the hydrogels applicability in tissue engineering. The obtained DD = 89% of the tested chitosan provided its good solubility and increased access to free amino groups, thus making the material more reactive to crosslinking process [27-29].

The obtained CS solutions before and after the single crosslinking process (after 3 days) are shown in FIG. 2. The addition of VAN solution caused an immediate colour change. This indicated the beginning of the reaction forming Shiff-base between the CS amino groups and the VAN aldehyde groups, as well as hydrogen bonds between both hydroxyl groups [30]. The higher the mass ratio of CS:VAN was, the more immediate the crosslinking process. In the 1.6van case, the solution viscosity was visibly higher after just one minute of mixing. The structure binded more quickly and externally more strongly, thus blocking the access to the material's deeper parts, which resulted in the insufficient crosslinking and the non-homogenous structure. Although mechanical properties increased with the increased amount of crosslinking agent, the chemical stability decreased [31].

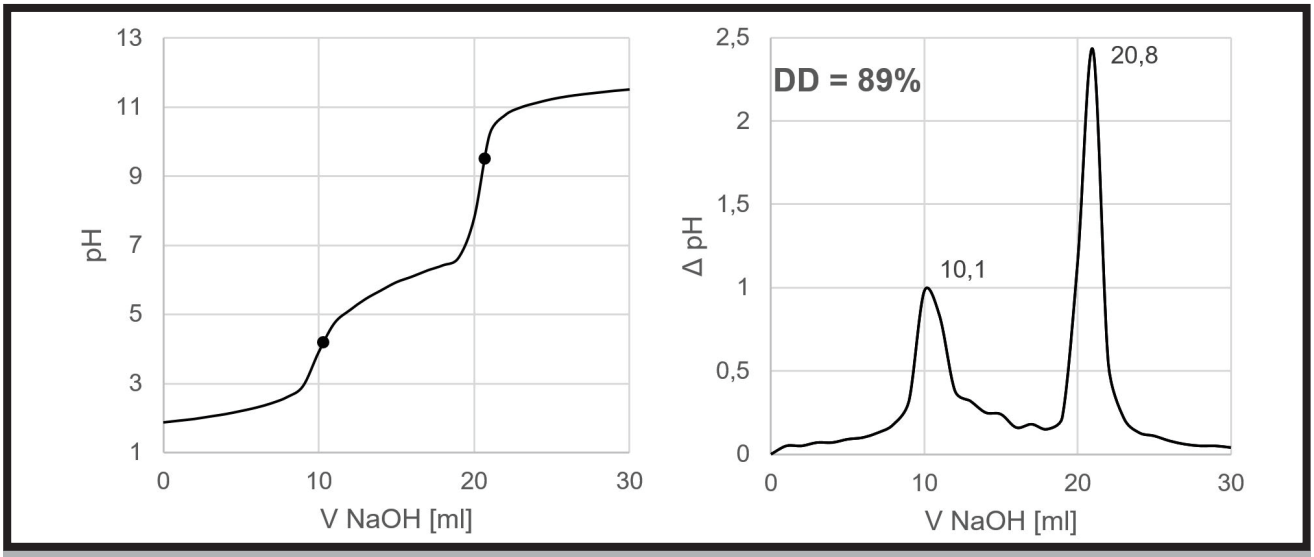

FIG. 1. The results of titration for CS powder – determination of deacetylation degree.

FIG. 2. The obtained samples before (left) and after (right) the single crosslinking, according to increasing mass ratio of VAN:CS.

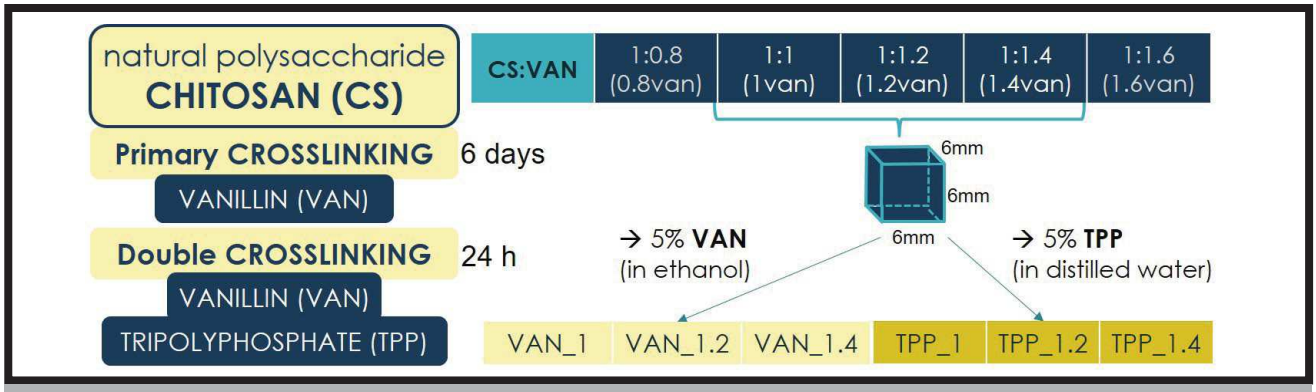

FIG. 3. The scheme of preparing the double crosslinked samples.

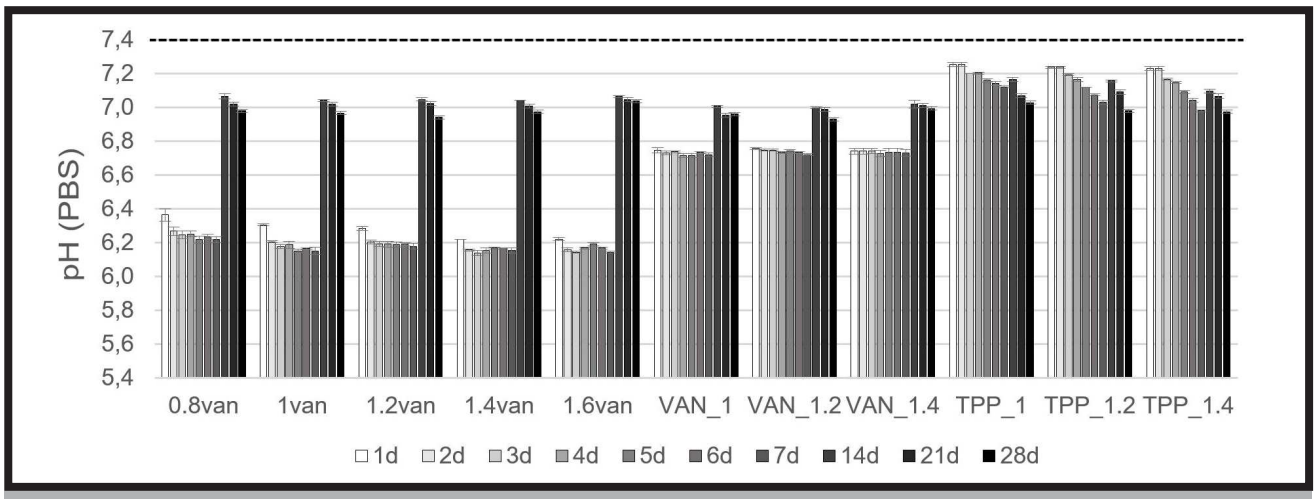

FIG. 4. The chemical stability test results – pH changes of incubation medium (PBS); triplets were tested for each type of material; error bars present standard deviations.

The scheme of double crosslinked materials preparation is presented in FIG. 3. The prepared cubic samples were maintained in the VAN or TPP solution for 24 h and then washed with distilled water for further studies. The TPP addition led to the formation of an ionically crosslinked hydrogel network, thus changing its properties [32].

The samples chemical stability was evaluated via the *in vitro* incubation test carried out in the PBS solution. The changes in the pH of the incubation media are presented in FIG. 4. The results indicated higher chemical stability of the double VAN-crosslinked samples, as compared to the single crosslinked CS. An initial pH decrease was caused by washing out the residual acetic acid or the non-crosslinked chitosan cations. However, the double VAN-crosslinked and TPP-crosslinked samples provided the initial pH at a higher level of about 6.7 and 7.2, respectively. The TPP addition in the hydrogel did not acidify the PBS solution significantly. Due to its alkaline nature [33], it provided better pH compatibility with human physiological fluids. However, for the TPP sample, its pH decreased with the incubation time as compared to the VAN-crosslinked sample where pH remained relatively constant both before and after the PBS substitution. Singh *et al.* provided evidence that the pH decrease and the lower oxygen level (2%) may affect cell viability and chondrogenesis [34]. The observations after 4 days of the cell culture pointed a decreased cell viability at pH = 6.2; the effect was not observed at pH = 7.2. Moreover, the alkaline environment seemed to be more beneficial for osteogenesis [35].

The results suggested that the double-crosslinked hydrogel network was more interconnected, the additional bonds formed and the stable structure was created. This initial effect was reduced after the PBS solution was substituted with the fresh one. This suggests that a longer pre-incubation period is required before testing the materials, for example in biological studies.

The weight loss results (FIG. 5) indicated that the double VAN-crosslinked samples exhibited the slowest degradation rate. The VAN_1, VAN_1.2 and VAN_1.4 samples after 4 weeks of the PBS incubation lost only around 5 % of their initial weight, while for the double TPP-crosslinked samples the weight loss was over twice as high. The weight loss of the single crosslinked samples was of 14-34%. In addition, starting with 1van, the more vanillin was in the material, the higher weight loss was observed. As mentioned above, the higher crosslinking agent/chitosan mass ratio was the more insufficient crosslinking occurred - as the non-crosslinked parts of the polymer dissolved and got released in the PBS solution. This phenomenon suggested that the optimal proportion of CS:VAN to crosslink the material in the first step would be 1:1. The second step of improving the crosslinked network provided a significantly more stable chemical structure, thus reducing the mass loss and slowing down the degradation process.

In general, it is necessary to apply chemically stable scaffolds to effectively repair tissue damages, otherwise, the regeneration process may be incomplete and new defects may occur. Therefore, it is vital to provide a relatively stable scaffold which may support tissue regeneration for minimum 3 months. During this period the scaffold integrates with the material and then the new tissue fills the defect. Moreover, the applied scaffold should degrade within 1 year [36,37].

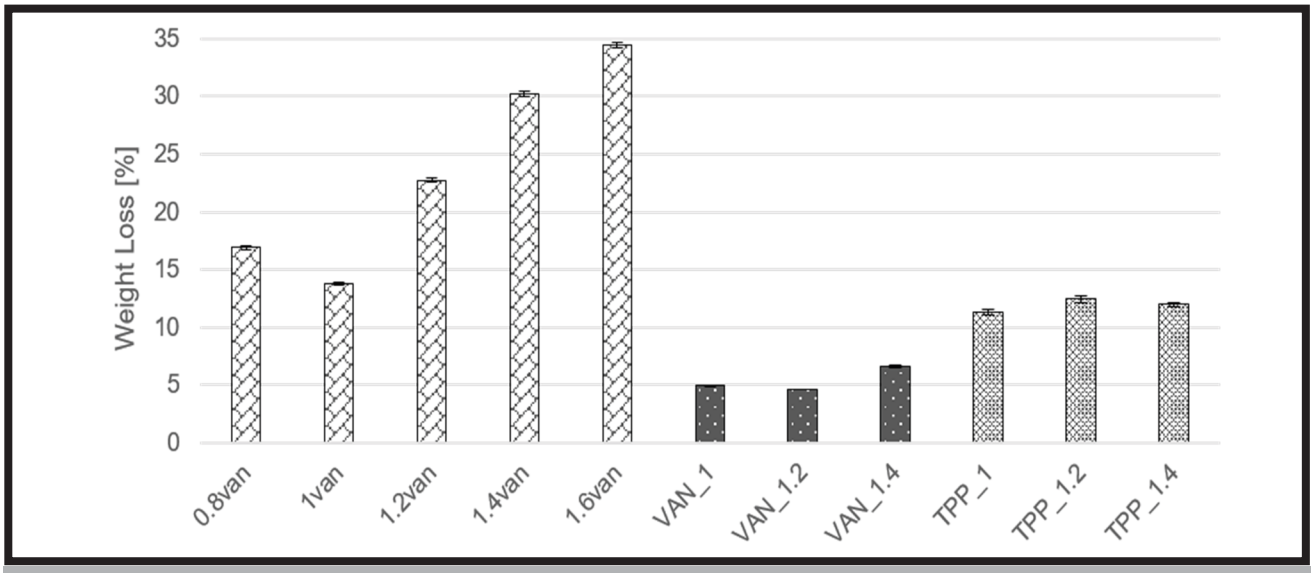

FIG. 5. The chemical stability test results – weight loss after 28 days of incubation in PBS solution; triplets were tested for each type of material; error bars present standard deviations.

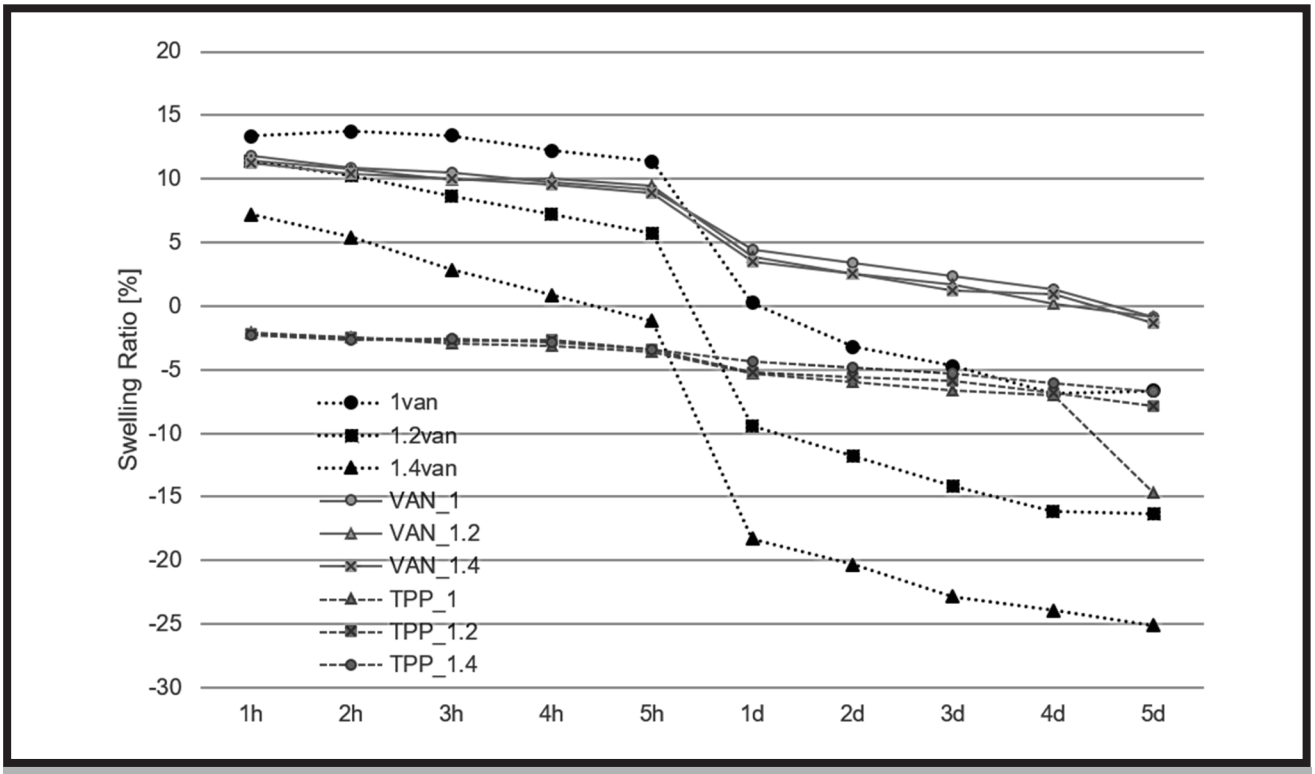

FIG. 6. The swelling ratio results after incubation in PBS solution; single samples were tested for each type of material.

The results of the swelling ratio during incubation in the PBS solution are presented in FIG. 6. The double VAN-crosslinking process had no significant effect on the initial SR in comparison to the single crosslinking – the swelling ratios for these materials were in the range of 7-13%. It was observed how the amount of vanillin added to the polymer solution affected the swelling process. The less vanillin was added, the higher SR was. The more elastic network and the lower amount of linkages facilitated the water absorption by the material. However, swelling of the single crosslinked samples decreased, reaching the negative values after 1 day of incubation. This may suggest the progressive degradation and the release of the non-crosslinked material. A significant difference was observed for the double crosslinked samples. The TPP-crosslinked samples did not swell and their SR was at a similar level. However, the slow degradation was observed – the obtained values changed during the incubation time from -2.5% to -7.5%. It is important to tailor the swelling character of the hydrogel scaffold for the proper cell migration through the network. Such a match makes it possible to control the transport of the ingredients necessary for tissue regeneration [38]. In addition, the inappropriately high swelling ratio may cause significant changes in the mechanical parameters, such as the chain straightening resulting in the modulus decrease [39].

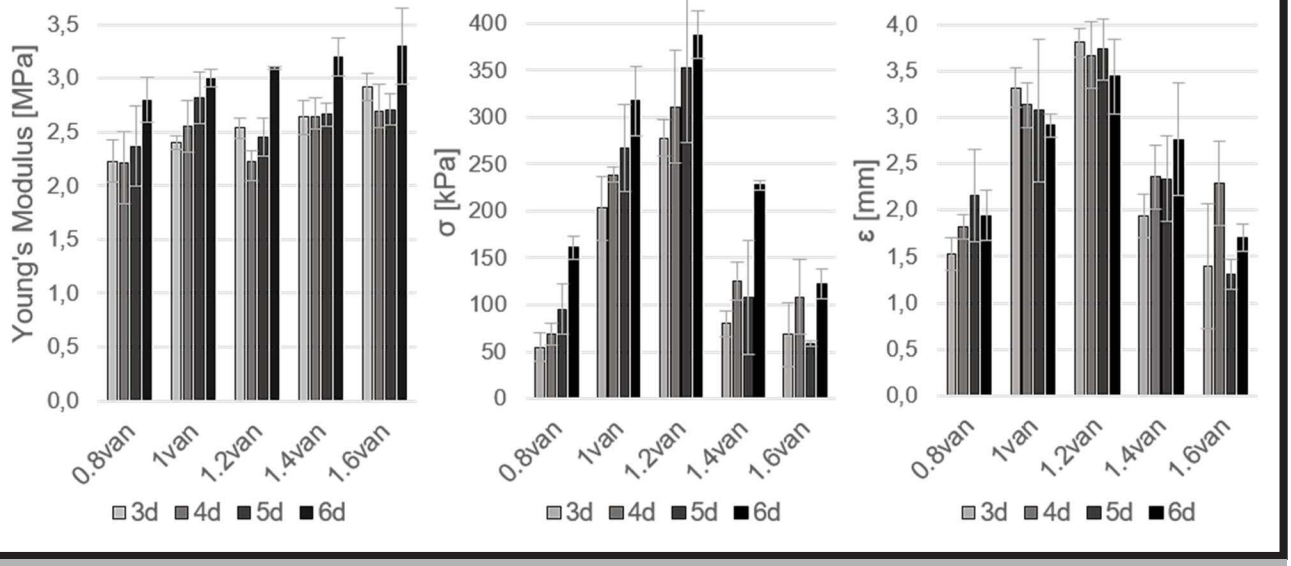

FIG. 7. The results of static compression test for the single crosslinked samples: Young's modulus, compression strength, strain; triplets were tested for each type of material; error bars present standard deviations.

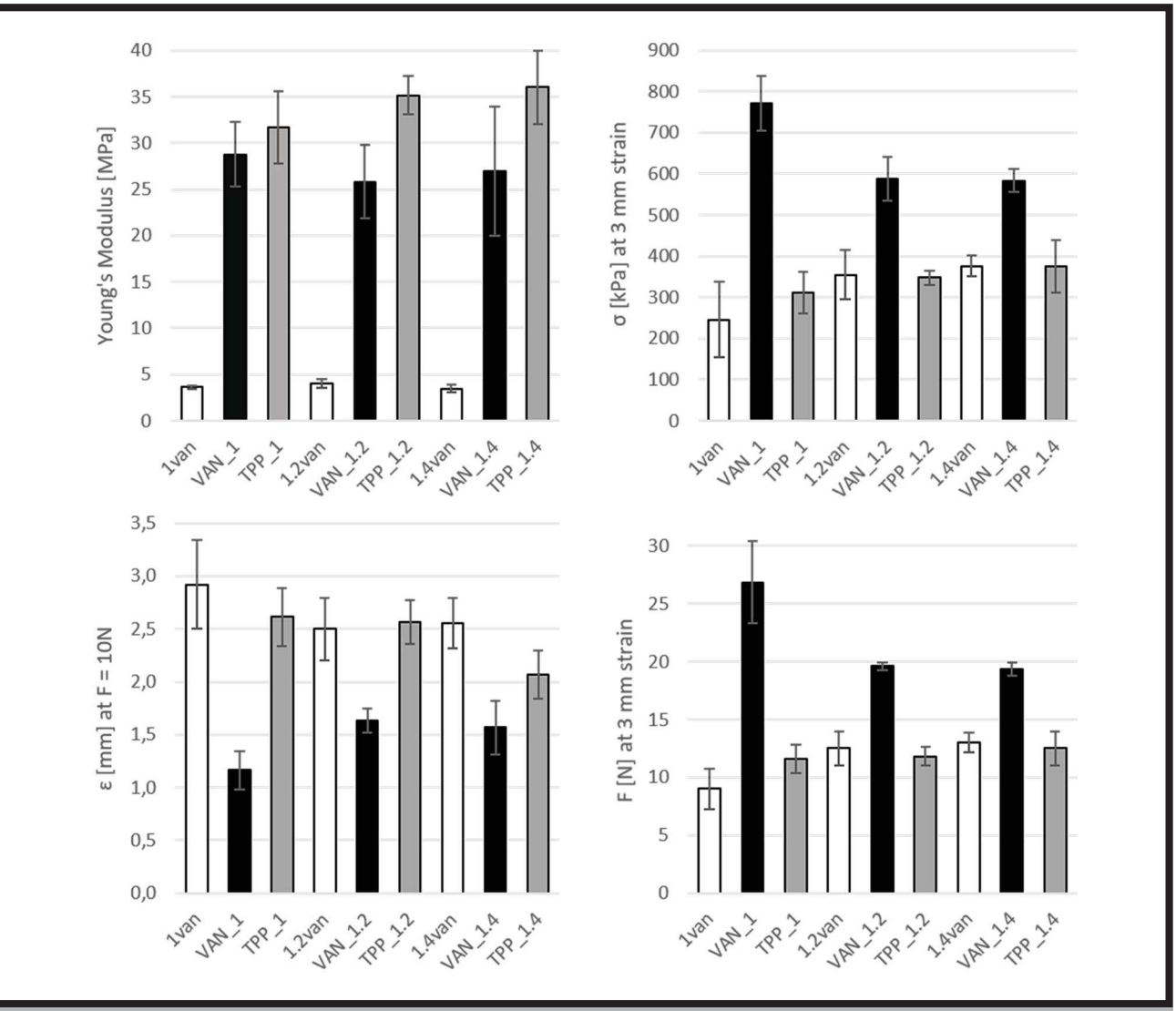

FIG. 8. The results after static compression test for the double crosslinked samples: Young's modulus, compression strength at 3 mm strain, stress at 3 mm strain, strain at stress F = 10 N; triplets were tested for each type of material; error bars present standard deviations.

The mechanical properties of the single crosslinked samples related to the initial crosslinking time are presented in FIG. 7. The Young's modulus values were similar for all the samples and increased slightly with the crosslinking time, reaching the maximum after the 6th day. However, both the compression strength and the strain values were the highest for the three middle samples, especially for 1.2van. The increase in brittleness for 1.4van and 1.6van resulted in lower compression strength and strain. The residues of unbound vanillin in these materials may have crystallized and acted like an additive making the material less flexible.

The static compression test was also carried out for the double crosslinked samples (FIG. 8). A significant increase in Young's modulus was observed for all the materials after the double crosslinking process, especially for the TPP samples. However, the double VAN samples displayed a more durable structure which resulted in higher compression strength and lower strain, especially for VAN_1.

In comparison to the single crosslinked samples, the double crosslinked ones showed elastic deformation during the compression test. The samples returned to their original shape after the test - in opposite to the single-crosslinked sample, which collapsed at the specific strain.

In general, hydrogels are characterized by poor mechanical properties. However, the hydrogels tested in this work showed efficient properties during compression tests. Both the Young's modulus and the compressive strength reached the values in the range of megapascals – it is very high when compared to the parameters obtained for natural polysaccharide hydrogels in the literature. Ahearne et al. described alginate and agarose-based hydrogels with the Young's modulus of about 14-15 kPa [40]. Wen et al. proposed chitosan-based hydrogels with high mechanical strength where the values were in the range of 25-200 kPa [41]. The CS-based hydrogels tested in this work exhibited the compression strength with the value of up to 800 kPa. Mirahmadi et al. obtained the chitosan/glycerophosphate/silk fibroin hydrogel with enhanced mechanical properties. DMA test results showed compressive modulus values in the range of 1.5-3.5 kPa [42].

Strong linkages between CS and vanillin provide a significant improvement of mechanical properties after the compression test. Both the Young's modulus and the compressive strength are crucial parameters with regard to tissue regeneration. What is more, mechanical parameters should be adjusted to the repaired tissue. For example, the maximum strength of hyaline cartilage is 1-18 MPa and elastic modulus 0.4-19 MPa [43]. Therefore, the double VAN-crosslinked hydrogels may support the cartilage regeneration due to the appropriate mechanical characteristics.

The compression test results for the double crosslinked samples after 28 days of incubation are shown in FIG. 9. The *in vitro* degradation process of the TPP samples may have weakened the materials. The Young's modulus decrease was of about 80% for the TPP sample, while for the VAN samples it was two-fold lower. Moreover, there was no significant change in the VAN samples' compression strength when comparing this property before and after incubation.

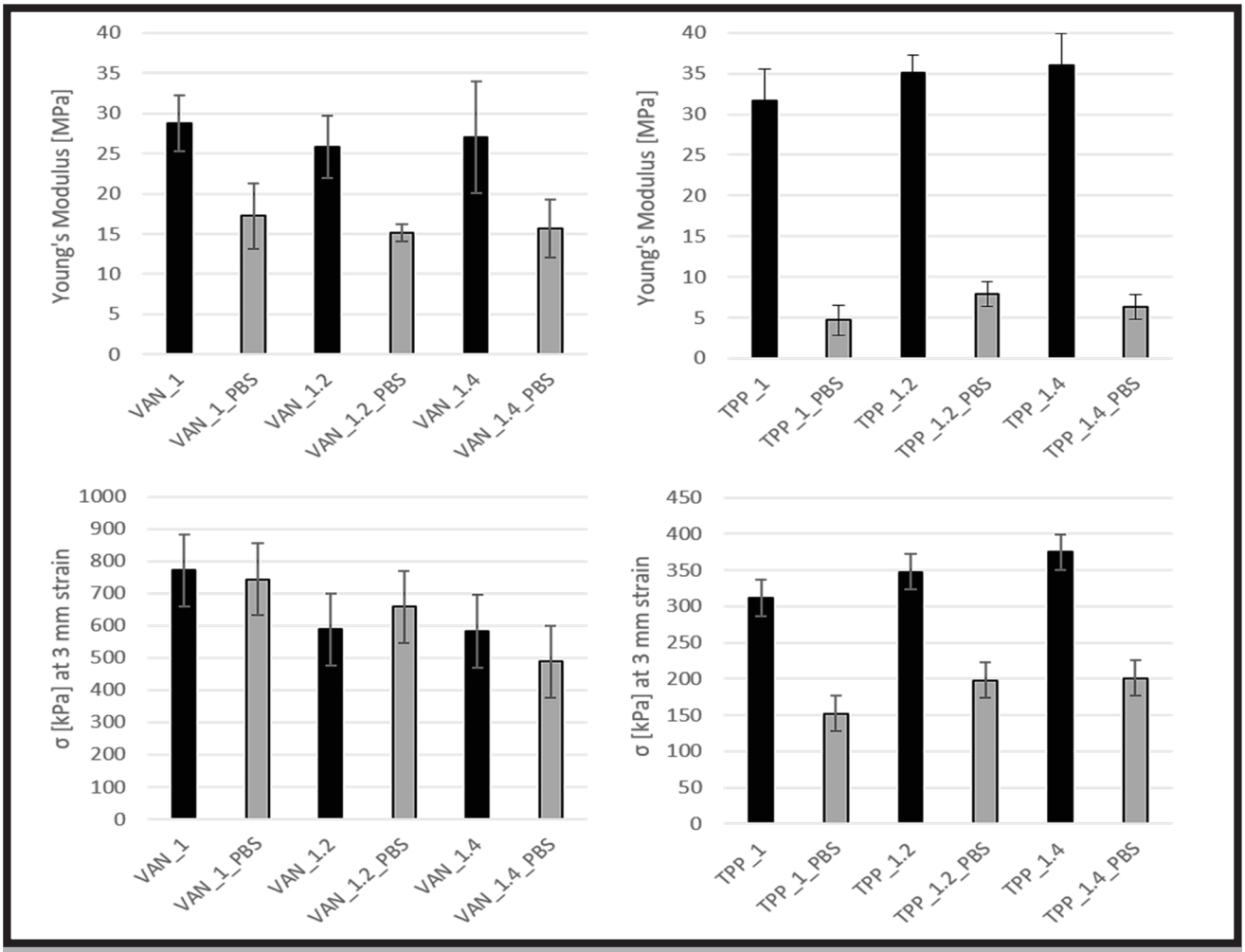

FIG. 9. The results of static compression test for double crosslinked samples before and after 4 weeks incubation in PBS solution; triplets were tested for each type of material; error bars present standard deviations.

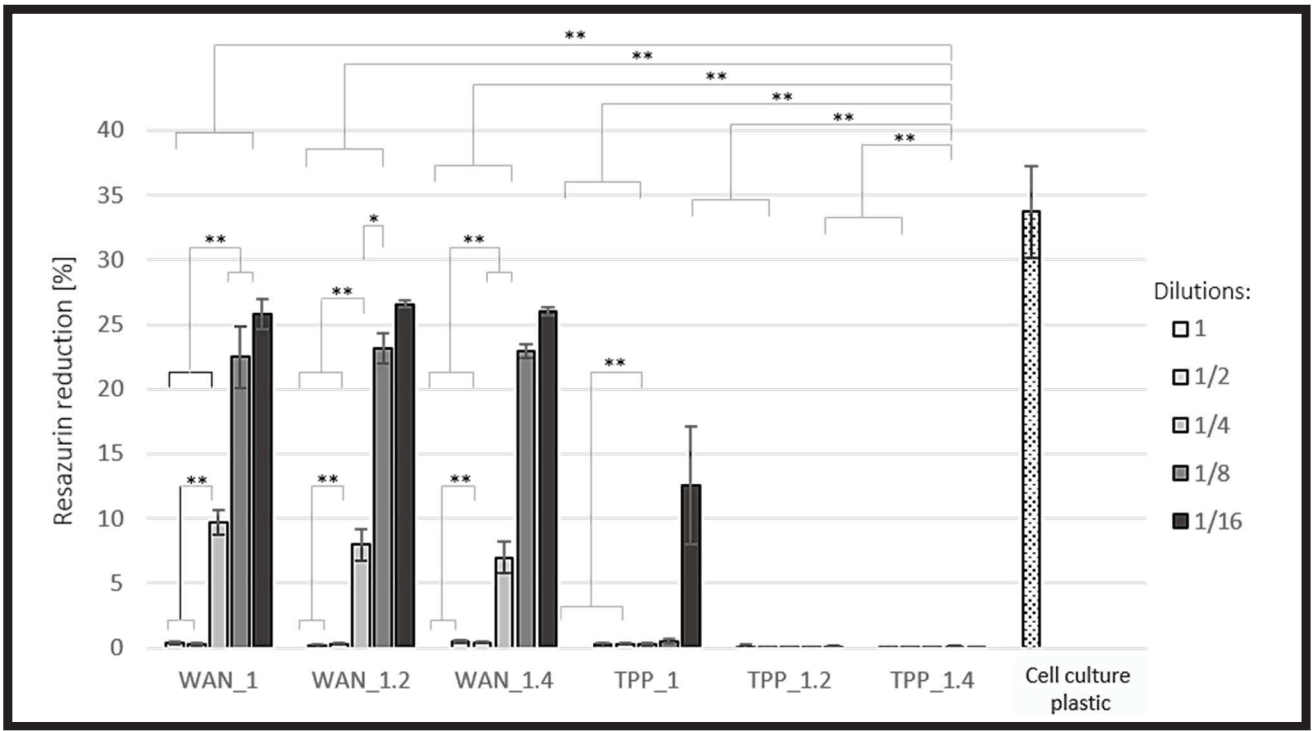

FIG. 10. Metabolic activity of cells cultured for 24 h in the presence of sample extracts in dilutions: 1 (undiluted extract), 1/2, 1/4, 1/8 and 1/16 (statistically significant differences at the level of *p <0.05, **p <0.001).

The tests proved that all the double VAN-crosslinked samples may constitute beneficial supports for tissue regeneration. As their mechanical properties are stable after 4 weeks of incubation in PBS, the materials may effectively support cell adhesion and proliferation, thus providing the complete sufficient reconstruction of the tissue [44].

FIG. 10 shows the results of the cell metabolic activity (reduction of resazurin) of the cultures incubated for 24 h in the presence of samples extracts at the following dilutions: 1 (undiluted extract), 1/2, 1/4, 1/8 and 1/16. These results were confirmed by the live/dead fluorescent staining test. The representative microscopic images of cells are shown in FIG 11.

The cells incubated in the pure culture medium showed the highest metabolic activity. All the cells visible were alive and retained the correct morphology typical for MG-63 cells (good adhesion, elongated spindle-shape). For all the samples, regardless of the dilution, the cell viability was lower than for the reference sample (pure culture medium). However, at the 1/4 dilution for the double VAN crosslinked samples the live cells were observed. The cells adhesion and proliferation may be limited by the residues of ethanol. The biodegradation results in the PBS solution suggested that a longer pre-incubation period might improve cell viability. Moreover, the higher concentrations of vanillin may reduce the cell viability, e.g. for the 50-200 μM vanillin concentration the lower cell viability was observed. Additionally, the 3-days pre-treatment of incubation in vanillin showed the significantly inhibited effect of the vanillin at the concentration over 5 μM on growth and the spheroid formation of NCI-H460 cells [45]. For all the TPP series samples, only the 1/16 dilution of TPP_1 showed a few live and flattened cells. For the TPP_1.2 and TPP_1.4 the cells revealed no metabolic activity (reduction of resazurin below 0.5%), which indicated the material's cytotoxicity after the double crosslinking with TPP.

Vanillin generally shows a low cytotoxicity effect, however, the crosslinker residues may be potentially toxic and to overcome this disadvantage the lower concentrations should be used. Zou *et al.* discussed the inflammation effect of vanillin and observed the mild inflammation at 4 weeks of the cell culture [46]. A similar effect was described for TPP by Gurses *et al*. With an increasing amount of sodium trypolyphosphate and sodium citrate, the significantly decreased human kidney cell viability was noticed [47]. Freitas Mariano *et al.* also described a negative influence of the TPP addition in the CS matrix on cell viability [48]. Therefore, it is necessary to determine the sufficient concentration of a crosslinking agent to reduce the cytotoxic effect of the hydrogel.

Conclusions

The double crosslinked chitosan hydrogels with the improved mechanical properties and chemical stability were obtained in this study with the use of vanillin as a natural crosslinking agent. The efficient mechanical properties and higher chemical stability prove high effectiveness of the double crosslinking process using vanillin. The results of the *in vitro* biological test revealed cytotoxicity of the double TPP crosslinked material. Although the polymer and the crosslinking agent used in the study are biocompatible, even a small amount of the solvent – ethanol – may be a barrier for cell proliferation. However, the cell viability could be improved, e.g. by a longer incubation time (to wash out the residues of cytotoxic solvents). Determining the sufficient concentration of the crosslinking agent should also be considered as an important aspect. Further studies are required to improve cytocompatibility of the testes hydrogels.

In the next step, the double VAN crosslinked hydrogels will be modified with additives, such as hydroxyapatite or graphene oxide to evaluate their influence on the material properties. Then, the choice of appropriate layers and optimization of the technology may result in mechanically stronger hierarchical structures dedicated for bone and cartilage tissue engineering.

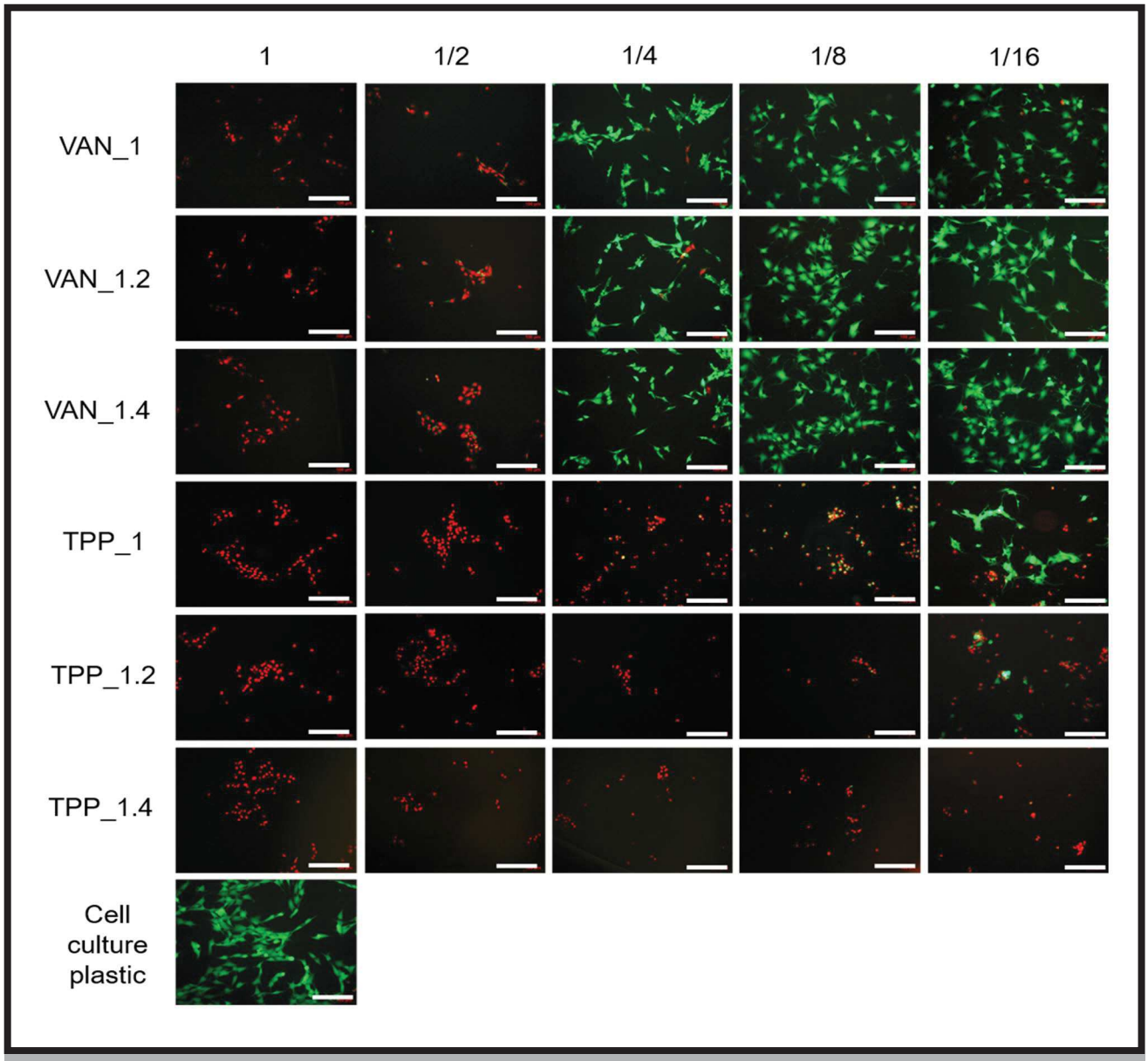

FIG. 11. Live/dead staining of cells cultured for 24 h in the presence of sample extracts in dilutions: 1 (undiluted), 1/2, 1/4, 1/8 and 1/16 (scale bar: 200 µm).

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References

[1] Croisier F., Jérôme C.: Chitosan-based biomaterials for tissue engineering. European Polymer Journal 49 (2013) 780–792.
[2] Koosha M., Raoufi M., Moravvej H.: One-pot reactive electrospinning of chitosan/PVA hydrogel nanofibers reinforced by halloysite nanotubes with enhanced fibroblast cell attachment for skin tissue regeneration. Colloids Surfaces B Biointerfaces 179 (2019) 270–279.
[3] Huang L. *et al.*: Antibacterial poly (ethylene glycol) diacrylate/chitosan hydrogels enhance mechanical adhesiveness and promote skin regeneration. Carbohydrate Polymers 225 (2019) 115110.
[4] Moreira C.D.F., Carvalho S.M., Sousa R.G., Mansur H.S., Pereira M.M.: Nanostructured chitosan/gelatin/bioactive glass in situ forming hydrogel composites as a potential injectable matrix for bone tissue engineering. Materials Chemistry and Physics 218 (2018) 304–316.
[5] Saekhor K. *et al.*: Preparation of an injectable modified chitosan--based hydrogel approaching for bone tissue engineering. International Journal of Biological Macromolecules 123 (2019) 167–173.
[6] Liu J., Yang B., Li M., Li J., Wan Y.: Enhanced dual network hydrogels consisting of thiolated chitosan and silk fibroin for cartilage tissue engineering. Carbohydate Polymers 227 (2020) 115335.
[7] Shao J., Ding Z., Li L., Chen Y., Zhu J., Qian Q.: Improved accumulation of TGF-β by photopolymerized chitosan/silk protein bio-hydrogel matrix to improve differentiations of mesenchymal stem cells in articular cartilage tissue regeneration. Journal of Photochemistry and Photobiology B: Biology 203 (2020) 111744.
[8] Jafarkhani M., Salehi Z., Nematian T.: Preparation and characterization of chitosan/graphene oxide composite hydrogels for nerve tissue Engineering. Materials Today: Proceedings 5 (2018) 15620–15628.
[9] Badhe R. V. *et al*.: A composite chitosan-gelatin bi-layered, biomimetic macroporous scaffold for blood vessel tissue engineering. Carbohydrate Polymers 157 (2017) 1215–1225.
[10] Ravishankar K., Dhamodharan R.: Advances in chitosan-based hydrogels: Evolution from covalently crosslinked systems to ionotropically crosslinked superabsorbents. Reactive and Functional Polymers 149 (2020) 104517.
[11] Mikhailov S. N. *et al.*: Crosslinking of Chitosan with Dialdehyde Derivatives of Nucleosides and Nucleotides. Mechanism and Comparison with Glutaraldehyde. Nucleosides, Nucleotides and Nucleic Acids 35 (2016) 114–129.
[12] Zhang Z.H. *et al.*: Enhancing mechanical properties of chitosan films via modification with vanillin. International Journal of Biological Macromolecules 81 (2015) 638–643.
[13] Stroescu M. *et al:* Chitosan-vanillin composites with antimicrobial properties. Food Hydrocolloids 48 (2015) 62–71.
[14] Tomadoni B., Ponce A., Pereda M., Ansorena M.R.: Vanillin as a natural cross-linking agent in chitosan-based films: Optimizing formulation by response surface methodology. Polymer Testing 78 (2019) 105935.
[15] Cestari A.R., Vieira E.F.S., Mattos C.R.S.: Thermodynamics of the Cu(II) adsorption on thin vanillin-modified chitosan membranes. The Journal of Chemical Thermodynamics 38 (2006) 1092–1099.
[16] Zou Q., Li J., Li Y.: Preparation and characterization of vanillin--crosslinked chitosan therapeutic bioactive microcarriers. International Journal of Biological Macromolecules 79 (2015) 736–747.
[17] Walke S. *et al*.: Fabrication of chitosan microspheres using vanillin/TPP dual crosslinkers for protein antigens encapsulation. Carbohydrate Polymers 128 (2015) 188–198.
[18] Berger J. *et al.*: Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. European Journal of Pharmaceutics and Biopharmaceutics 57 (2004) 19–34.
[19] Guaresti O. *et al.*: Synthesis and characterization of a biocompatible chitosan–based hydrogel cross–linked via 'click' chemistry for controlled drug release. International Journal of Biological Macromolecules 102 (2017) 1–9.
[20] Gohil S.V. *et al*.: Evaluation of enzymatically crosslinked injectable glycol chitosan hydrogel. Journal of Materials Chemistry B 3 (2015) 5511–5522.
[21] Sun Z., *et al.*: Multistimuli-Responsive, Moldable Supramolecular Hydrogels Cross-Linked by Ultrafast Complexation of Metal Ions and Biopolymers. Angewandte Chemie International Edition 54 (2015) 7944–7948.
[22] Takei T. *et al.:* Autoclavable physically-crosslinked chitosan cryogel as a wound dressing. Journal of Bioscience and Bioengineering 125 (2018) 490–495.
[23] Smith R. A., Walker R. C., Levit S. L., Tang C.: Single-step self-assembly and physical crosslinking of PEGylated chitosan nanoparticles by tannic acid. Polymers (Basel) 11 (2019) 749.
[24] Sacco P. *et al.*: Insight into the ionotropic gelation of chitosan using tripolyphosphate and pyrophosphate as cross-linkers. International Journal of Biological Macromolecules 92 (2016) 476–483.
[25] Csaba N. *et al.*: Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery. International Journal of Pharmaceutics 382 (2009) 205–214.
[26] Czechowska-Biskup R. *et al.*: Determination of degree of deacetylation of chitosan - Comparision of methods. Progress on Chemistry and Application of Chitin and its Derivatives 17 (2012) 5–20.
[27] Kosheleva R., *et al.*: Effect of grafting on chitosan adsorbents. Composite Nanoadsorbents, Elsevier, Oxford, U.K., 2018, 49-66.
[28] Aizat M. A., Aziz F.: 12 - Chitosan Nanocomposite Application in Wastewater Treatments. Nanotechnology in Water and Wastewater Treatment: Theory and Applications, Elsevier, 2018, 243–265.
[29] Lv S. H.: High-performance superplasticizer based on chitosan," in Biopolymers and Biotech Admixtures for Eco-Efficient Construction Materials, Elsevier Inc., 2016, 131–150.
[30] Xu C., Zhan W., Tang X., Mo F., Fu L., Lin B.: Self-healing chitosan/vanillin hydrogels based on Schiff-base bond/hydrogen bond hybrid linkages. Polymer Testing 66 (2018) 155–163.
[31] Ozbolat I. T.: The Bioink∗∗With contributions by Monika Hospodiuk and Madhuri Dey, The Pennsylvania State University, 3D Bioprinting, Elsevier, 2017, 41–92.
[32] Jóźwiak T. *et al.*: Effect of ionic and covalent crosslinking agents on properties of chitosan beads and sorption effectiveness of Reactive Black 5 dye. Reactive and Functional Polymers 114 (2017) 58–74.
[33] Hourant P.: General Properties of the Alkaline Phosphates: - Major Food and Technical Applications, Phosphorus Research Bulletin 15 (2004) 85–94.
[34] Singh S.: Effects of different pH and oxygen levels on proliferation and chondrogenic differentiation of human mesenchymal stem cells cultured in hydrogels, MSc thesis, Department of Applied Physics, Chalmers University of Technology, Gothenburg, Sweden 2014
[35] Galow A.M. *et al.*: Increased osteoblast viability at alkaline pH in vitro provides a new perspective on bone regeneration. Biochemistry and Biophysics Reports 10 (2017) 17–25.
[36] Nooeaid P., Salih V., Beier J. P., Boccaccini A. R.: Osteochondral tissue engineering: Scaffolds, stem cells and applications. Journal of Cellular and Molecular Medicine 16 (2012) 2247–2270, 2012.
[37] Xiao H. *et al.*: Osteochondral repair using scaffolds with gradient pore sizes constructed with silk fibroin, chitosan, and nano-hydroxyapatite. International Journal of Nanomedicine 14 (2019) 2011–2027.
[38] Zhu J., Marchant R.E.: Design properties of hydrogel tissue-engineering scaffolds, Expert Review of Medical Devices 8 (2011) 607–626.
[39] Young S.A. *et al.*: In situ -forming, mechanically resilient hydrogels for cell delivery. Journal of Materials Chemistry B 7 (2019) 5742–5761.
[40] Ahearne M. *et al.*: Mechanical Characterisation of Hydrogels for Tissue Engineering Applications Hydrogels for Tissue Engineering, 2008.
[41] Wen Y., Li F., Li C., Yin Y., Li J.: High mechanical strength chitosan-based hydrogels cross-linked with poly(ethylene glycol)/polycaprolactone micelles for the controlled release of drugs/growth factors. Journal of Materials Chemistry B 5 (2017) 961–971.
[42] Mirahmadi F. *et al.*: Enhanced mechanical properties of thermosensitive chitosan hydrogel by silk fibers for cartilage tissue engineering. Materials Science and Engineering: C 33 (2013) 4786–4794.
[43] Silver F.H. *et al.*: Mechanical Properties of Tissues, Biomaterials Science and Biocompatibility, Springer New York, 1999, 187–212.
[44] Ansari S. *et al.*: Engineering of gradient osteochondral tissue: From nature to lab. Acta Biomaterialia 87 (2019) 41–54.
[45] Srinual S. *et al.*: Suppression of cancer stem-like phenotypes in NCI-H460 lung cancer cells by vanillin through an Akt-dependent pathway. International Journal of Oncology 50 (2017) 1341–1351.
[46] Zou Q., Li J., Li Y.: Preparation and characterization of vanillin--crosslinked chitosan therapeutic bioactive microcarriers. International Journal of Biological Macromolecules 79 (2015) 736–747.
[47] Gurses M. S., Erkey C., Kizilel S., Uzun A.: Characterization of sodium tripolyphosphate and sodium citrate dehydrate residues on surfaces. Talanta 176 (2018) 8–16.
[48] Freitas Mariano K.C. *et al.*: Influence of chitosan-tripolyphosphate nanoparticles on thermosensitive polymeric hydrogels: structural organization, drug release mechanisms and cytotoxicity. International Journal of Polymeric Materials and Polymeric Biomaterials 69 (2020) 592–603.