Bone-Mimicking Injectable Gelatine/Hydroxyapatite Hydrogels

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Bioactive synthetic hydrogels have emerged as promising materials because they can provide molecularly tailored biofunctions and adjustable mechanical properties. To mimic the mineralogical and organic components of the natural bone, hydroxyapatite and a tyramine conjugate of gelatine were combined in this study. The effect of various amounts of *in situ* synthesized hydroxyapatite in gelatine-tyramine on the morphology and physical properties of injectable hydrogels was investigated. Mineralogical identification confirmed successful precipitation of *in situ* formed hydroxyapatite. Better distribution of hydroxyapatite crystal agglomerates within modified gelatine was found at 5 % of hydroxyapatite, which could be responsible for increased storage modulus with respect to pure gelatine hydrogel. Prepared composite hydrogels are non-toxic and support the proliferation of Hek293 cells.

Keywords:
gelatine, hydroxyapatite, injectable, hydrogel, rheology, cytotoxicity

Introduction

Hydrogels (i.e., three-dimensional cross-linked hydrophilic polymer networks containing a high quantity of water), have received considerable attention in the past decades in a wide range of applications. They possess a degree of flexibility very similar to natural tissue due to their large water content[1]. In the swollen state, hydrogels are soft and rubbery, resembling the living tissue, which opens up many opportunities for biomedical aplications. In particular, *in situ* gelling hydrogels have attracted attention for drug delivery application and tissue regeneration[2]. *In situ* gelling hydrogels are attractive because they can fill any shape of a defect, allow homogeneous incorporation of therapeutic molecules or cells, and require no surgical procedures for implantation[3].

Gelatine-based hydrogels have been extensively studied due to their low cost, biodegradability, biocompatibility, and non-immunogenic properties. A gelatine physical hydrogel is formed at temperatures below 25 °C. When the temperature is raised above approximately 30 °C, gelatine is in the liquid state, thus limiting its use in tissue regeneration. To avoid dissolution at body temperature, chemical crosslinkers as glutaraldehyde, genipin, carbodiimides, etc., have been used. A drawback of those crosslinkers is their potential cytotoxicity. Recently, gelatine-tyramine conjugates have been proposed as injectable hydrogels as they form stable hydrogels under a non-cytotoxic enzymatically catalysed reaction in the presence of small amounts of hydrogen peroxide[3].

Modified gelatine using tyramine grafting has been mostly used as a soft tissue substituent encapsulating cells, mainly for cartilage defects. Several works have been focused on improving the mechanical and biological properties of gelatine-tyramine based hydrogels, introducing polymer fibres[4] or other natural polymers, such as hyaluronic acid[5,6]. Poveda-Reyes *et al.*[4] modified gelatine-tyramine enzymatically crosslinked hydrogel by adding PHEMA-grafted PLLA fibres, which resulted in almost 3-fold increase in shear storage modulus. The PHEMA-grafting of PLLA ensured good interfacial interactions between PLLA microfibers and gelatine matrix without affecting the enzymatic crosslinking of the hydrogel.

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The same group[6] combined tyramine conjugates of gelatine and hyaluronic acid, in different proportions, in order to mimic extracellular matrix. Depending on the gelatine/hyaluronic acid ratio, injectable hydrogels showed improved mechanical stability and enhanced expression of aggrecan, collagen type II and GAG of encapsulated human mesenchymal stem cells without using the supplement of growth factors.

The enzymatic crosslinking of gelatine-tyramine has proved to be suitable for cell encapsulation and regeneration of soft tissues like cartilage; however, few attempts have been made in the modification of gelatine-tyramine osteoconductive properties using bioactive inorganic components (such as calcium phosphates). Bone tissue is a composite material consisting of organic (mostly collagen) and inorganic (calcium phosphates, mostly hydroxyapatite) components. To mimic the natural bone, Thinh *et al.*[7] prepared injectable hydrogels based on modified chitosan (chitosan-4 hydroxyphenylacetamide acid), gelatine-tyramine, and biphasic calcium phosphate nanoparticles (BCP NPs). Those gels exhibited very fast gelation ranging from 35 to 80 seconds, depending on the concentration of H_2O_2. They reported slower *in vitro* biodegradation in the presence of 10 % of BCP NPs within composite hydrogel. The same group[8] introduced calcium phosphate nanoparticles in gelatine-PEG modified hydrogel in order to enhance hydrogels bioactivity and biodegradability by varying the amount of BCP. They reported that higher surface roughness, originated from BCP, stimulates the attachment of MG-63 cells.

However, those gelatine-based hydrogels loaded with BCP nanoparticles were obtained by conventional mechanical mixing of previously synthesized inorganic phase. The blending of particles into polymer usually results in particle agglomeration due to the higher cohesion, which affects the final mechanical and surface properties. Moreover, it is difficult to avoid the precipitation of mixed inorganic particles previously to gelatine crosslinking.

According to the literature, no attempts have been made in developing an injectable gelatine/HAp material. Bearing this in mind, we proposed injectable gelatine-based composite hydrogels with *in situ* formed hydroxyapatite (HAp; $Ca_{10}(PO_4)_6(OH)_2$), mimicking the nanocrystallinity of natural tissue. To our knowledge, this is the first time to synthesize a nanostructured gelatine-tyramine/HAp injectable hydrogel via *in situ* precipitation of hydroxyapatite. It is hypothesized that functional carboxylic groups of gelatine could serve as specific nucleation sites for guided apatite crystallisation, leading to a formation of homogeneous nanostructured composite material. To avoid possible toxicity of final hydrogel, calcium carbonate as a non-toxic calcium precursor for HAp, would provide *in situ* synthesis of a more bioactive apatite phase, similar to the biological one (carbonated hydroxyapatite). Therefore, different gelatine/HAp hydrogels were prepared in order to investigate the optimal composition in terms of HAp particles distribution within the hydrogel.

Materials and methods

Preparation of tyramine conjugated gelatine

The gelatine (from porcine skin type A, gel strength 300, 80 mmol of COOH per 100 g of gelatine; Sigma-Aldrich, Germany) was modified by grafting the amino groups of tyramine (tyramine hydrochloride, ≥98 %; Sigma-Aldrich, Germany) to the carboxylic groups of gelatine, via the carbodiimide-mediated condensation, as shown in Fig. 1. The molar ratios for the grafting were tyramine/*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC; Iris Biotech GmbH, Marktredwitz, Germany)/COOH = 2/2/1 and *N*-hydroxysuccinimide (NHS, 98 %; Sigma-Aldrich, Germany)/EDC = 1/10. Briefly, 3 % (w/v) of gelatine was dissolved in 50 mmol L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES, >99 %; Sigma-Aldrich, Germany) at 60 °C for 30 min under stirring (0.6 g of gelatine and 0.195 g of MES in 20 mL). An amount of 0.111 g of tyramine hydrochloride was then added and the mixture was stirred for 20 min at room temperature. The pH was adjusted to 6. After that, 7 mg of NHS was added and homogenised by stirring for 30 min. Finally, 123 mg of EDC was added to the mixture and stirred for another 24 h at 37 °C. Unreacted reagents were removed via dialysis against deionised water for 48 h. The modified gelatine was frozen under liquid nitrogen and kept overnight at –80 °C. Frozen gelatine was then lyophilised under vacuum (p < 1 mbar) at –100 °C for two days. Lyophilised gelatine was denoted as 'Gel-Tyr'.

Synthesis of hydroxyapatite particles as a control material

Hydroxyapatite particles were synthesized as a control for further identification and characterisation. Hydroxyapatite was prepared by the wet chemical precipitation method using calcite ($CaCO_3$; TTT, Croatia) and urea phosphate ($(NH_2)_2CO$–H_3PO_4, UPH; Aldrich Chemistry, USA) as precursor materials. Specific amount of calcite as a source of calcium ions was added to 100 mL of distilled water followed by the addition of UPH with respect to the Ca/P ratio of 1.67. Temperature was set at 50 °C

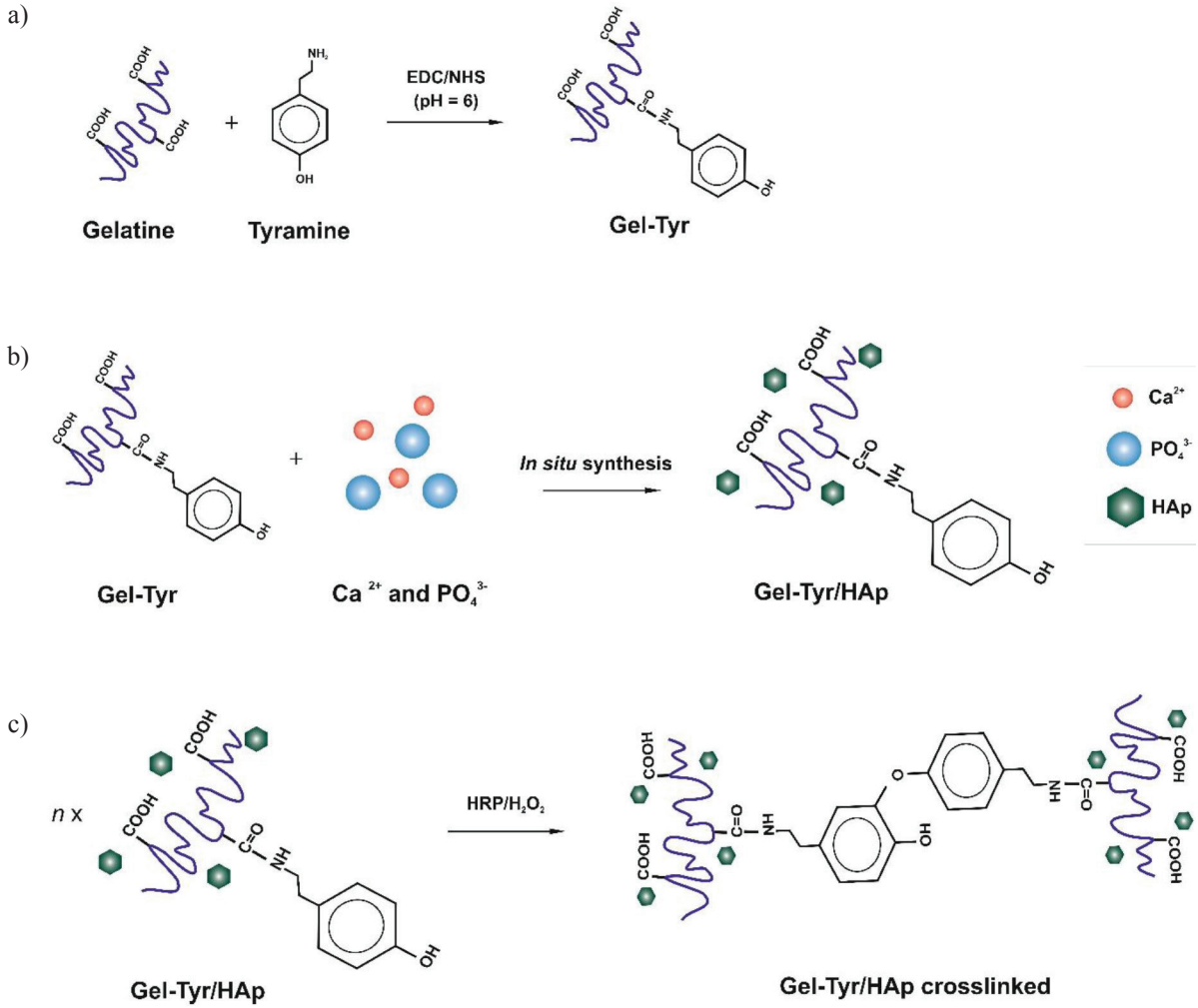

Fig. 1 – *Illustration of hydrogel synthesis performed in three steps: a) tyramine grafting on carboxylic group of gelatine; b) in situ precipitation of hydroxyapatite within Gel-Tyr solution from calcium and phosphate precursors; c) enzymatic crosslinking of Gel-Tyr/HAp composite using horseradish peroxidase with hydrogen peroxide*

and stirring continued for 4 days. Obtained precipitate suspension was filtered, washed three times with distilled water, and left to dry at room temperature.

In situ synthesis of hydroxyapatite in tyramine modified gelatine solution

The gelatine-tyramine/hydroxyapatite (Gel-Tyr/HAp) composite was prepared by the *in situ* wet precipitation method. Starting 3 % (*w*/*v*) Gel-Tyr solution was prepared by dissolving gelatine-tyramine lyophilised powder in deionised water (pH of 7) at 37 °C. The specific amount of calcite was then added into the polymer solution with rigorous stirring at 1700 rpm at 37 °C. When homogeneous gelatine-tyramine/calcite suspension was achieved, appropriate amount of UPH with respect to the Ca/P ratio of 1.67 was added into the mixture. The reaction temperature was set at 50 °C, and the solution was stirred for 24 hours. The final suspension was cooled to room temperature, and subsequently frozen with liquid nitrogen, stored at –80 °C overnight, and lyophilised according to the previously described procedure. The hydroxyapatite content in final Gel-Tyr/HAp composites was set to 2, 5, and 10 % (w/w), and composites were denoted as 2 % HAp, 5 % HAp, and 10 % HAp, respectively.

Injectable composite hydrogel preparation

To prepare the composite gelatine-tyramine/hydroxyapatite hydrogel, enzymatic crosslinking was performed (Fig. 1) using 20 U mL^{-1} of horseradish peroxidase (HRP, type I, Sigma-Aldrich, Germany) (1:10 ratio of HRP: hydrogel volume), and 10 mmol L^{-1} H_2O_2 solution (Sigma-Aldrich, Germany) (1:10 ratio of HRP: hydrogel volume). The mixture was then quickly stirred (5 sec at 1000 rpm on Vortex) to obtain a homogeneous solution which allowed formation of a hydrogel at room temperature in a few minutes. After crosslinking, the samples were

washed with deionised water, lyophilised according to the previously described procedure, and stored for further characterisation.

Characterisation of materials

Proton nuclear magnetic resonance and UV spectrophotometer

The measurements were performed as a part of previous research[4]. ^{1}H-NMR measurements were performed in D_2O for the gelatine and the tyramine grafted gelatine at a concentration of 10 mg mL^{-1} in a Spectra spin400 Ultra shield from Bruker. To determine the quantity of phenol (Ph) groups introduced in the gelatine, the absorbance of a 0.1 % (w/w) aqueous solution of the grafted gelatine was analysed at 275 nm with a CECIL CE9200 UV/VIS double beam spectrophotometer (Buck Scientific, Norwalk, USA).

Fourier transform infrared spectroscopy

All prepared samples were characterised using Fourier transform infrared spectroscopic analysis (FTIR). FTIR spectra were recorded by ATR-FTIR Bruker Vertex 70 spectrometer with a diamond crystal, 16 times in the absorption mode in the range of 4000 – 400 cm^{-1} with a resolution of 4 cm^{-1} at 20 °C.

X-ray diffraction analysis

The mineralogical composition of prepared materials was determined by X-ray diffraction analysis (XRD) on a Shimadzu XRD 6000 instrument with Cu K_a radiation at 40 kV and 30 mA, at angle range of $5° < 2\theta < 70°$, and with a scan rate of 0.2° s^{-1}. The identification of the crystal phase was carried out using ICDD database (The International Centre for Diffraction Data).

Scanning electron microscopy

Prior to imaging, all samples were shock-frozen in liquid nitrogen, quickly transferred to a freeze-drier, and lyophilised for 72 h. The morphology of lyophilised hydrogels was imaged by scanning electron microscopy (SEM) on a TESCAN Vega3SEM EasyProbe microscope with electron beam energy of 10 kV. Prior to scanning, the samples were sputtered with gold and palladium for 120 seconds.

Equilibrium water content

The swelling capacity of hydrogels was evaluated in deionised water. Crosslinked samples were immersed in deionised water for 2 h at room temperature. The swollen hydrogels were removed from the immersion medium, the water excess was absorbed by a filter paper, and hydrogels were weighed (w_{wet}). Swollen samples were frozen in liquid nitrogen, stored at –80 ºC overnight, and lyophilised to determine their dry weight (w_{dry}). The equilibrium water content (EWC) was determined by Equation 1:

$$\mathrm{EWC}(\%) = \frac{w_{wet} - w_{dry}}{w_{dry}} \cdot 100 \qquad (1)$$

Rheological properties

The rheological characterisation of hydrogels was carried out by a Discovery HR-2 Hybrid Rheometer (TA Instruments) using a parallel plates geometry of 20 mm diameter, and a gap between the plates of about 900 μm. The measurements were taken at 25 °C using a solvent trap to avoid water evaporation from the hydrogels during the experiment.

An amplitude test of crosslinked hydrogel was carried out at deformation range of 0.01 to 100 % and frequency of 1.26 rad s^{-1} to determine linear viscoelastic region of the hydrogels where the storage (G') and loss (G'') moduli are independent of the strain amplitude.

The time dependence of the shear storage and loss modulus was evaluated on the *in situ* gelation of the hydrogel at shear deformation of 1 % and an angular frequency of ω = 1.26 rad s^{-1} for 20 min to determine the influence of HAp content on gelation time of hydrogels.

A constant strain (1 %) frequency sweep test within the angular frequency range of 0.1 – 800 rad s^{-1} was performed on the *in situ* crosslinked hydrogels to determine the mechanical properties of the different hydrogels.

Cytotoxicity of hydrogels

The cytotoxicity of composite hydrogels was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5--diphenyltetrazolium bromide (MTT) assay on human embryonic kidney (Hek293) cell line. Prior to the assay, samples were disinfected with penicillin/streptomycin and antifungal agent (3 %) for 2 h at 4 °C. After disinfection, samples were washed three times in DPBS (Dulbecco's phosphate saline, pH 7.4), and subsequently incubated with culture medium (DMEM) supplemented with 10 % of fetal bovine serum (FBS) and 1 % of penicillin/streptomycin (P/S) for 24 h at 4 °C.

Meanwhile, Hek293 cells were seeded in a 96-well plate (Sarsted) in triplicates at a density of $3 \cdot 10^3$ cells/200 μL, and grown in Dulbecco's modified Eagle medium with 4500 mg L^{-1} glucose (DMEM-high glucose, Lonza) containing 10 % fetal bovine serum (FBS, Gibco), 100 U mL^{-1} peni-

cillin, and 100 mg mL^{-1} streptomycin (Sigma-Aldrich). After 1 day of culture, medium was replaced with extract from the samples, and incubated for next 3 days in 5 % CO_2 humidified atmosphere at 37 °C. Following the incubation period of 24 and 72 h, respectively, the medium was aspirated and 200 μL of MTT (Sigma-Aldrich) was added to each well at a concentration of 0.5 mg mL^{-1}. After 4 h of incubation at 37 °C, MTT was aspirated and formazan crystals were dissolved by adding 170 μL of DMSO to each well. After 1 h at room temperature, 100 μL of solution per well was transferred into clean 96-well plate to measure the absorbance on a microplate reader (Promega) at 570 nm. The cell viability was calculated with respect to the control (non-treated cells).

Statistical analysis of data

All experiments were performed in triplicate, and results are expressed as mean value corrected by the standard deviation. Statistical analysis of the data was carried out by one-way ANOVA test. Statistical significance between two groups was considered at a $p < 0.05$.

Results and discussion

Materials identification

The grafting of tyramine onto the gelatine macromolecules was verified using ^{1}H-NMR analysis. The presence of distinctive peaks for both aromatic ring protons (at 7.0 and 6.7 pmm) in Gel-Tyr spectrum indicated successful grafting (data not shown). This indication was also confirmed by the UV spectrophotometry with $1.9 \cdot 10^{-7}$ tyramine grafted groups per mg of gelatin[4]. The same gelatine was used in this study to prepare Gel-Tyr/HAp composite hydrogels.

The prepared gelatine-tyramine/hydroxyapatite (Gel-Tyr/HAp) composites and hydroxyapatite particles as a control material were characterised by FTIR and XRD analyses.

The absorption bands specific for the modified gelatine and hydroxyapatite are depicted in Fig. 2. The characteristic phosphate bands of hydroxyapatite control are found at 1029 cm^{-1}, which corresponds to the asymmetric stretching, band at 962 cm^{-1} assigned to the symmetric stretching and bands at 599 and 561 cm^{-1} were attributed to asymmetric bending vibrations of the PO_4^{3-} group[9]. The absorption bands specific for carbonate groups found at 1450 and 1421 cm^{-1} indicate the B-type substitution of CO_3^{2-} ions in hydroxyapatite structure. Moreover, the band at 873 cm^{-1} (HPO_4^{2-} group) also indicates the formation of non-stoichiometric calcium-deficient hydroxyapatite (Ca/P ≠ 1.67) with proposed structure $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$[10,11]. Stoichiometric HAp (Ca/P = 1.67) is the most used calcium phosphate in the development of bone tissue substitute materials[12]. However, its stoichiometry favours high thermal and chemical stability, which mostly results in slow bioresorption under physiological conditions. Natural bone tissue possesses calcium phosphates substituted with different monovalent and divalent cations and anions (Na^+, K^+, Mg^{2+}, Sr^{2+}, HCO_3^-, CO_3^{2-}, HPO_4^-, F^-). Carbonate ions are crucial in metabolic processes during formation of new bone, promoting faster calcium and phosphate diffusion from the hydroxyapatite lattice that are necessary in the later stage of bone mineralisation. Bearing this in mind, synthesized CO_3^{2-} substituted hydroxyapatite would show greater bioactivity and osteoconductivity under *in vivo* conditions than stoichiometric apatite.

The composite systems prepared with 2, 5, and 10 % of HAp show characteristic phosphate bands at 1029, 597 and 559 cm^{-1} with a slight shift of the phosphate asymmetric bending vibrations. Such phenomenon could indicate possible electrostatic interactions between *in situ* precipitated HAp and functional groups of gelatine-tyramine. As seen from Fig. 2, the intensity of phosphate bands increases with the amount of *in situ* formed HAp, with the strongest intensities found in the 10 % HAp composite.

Apart from the characteristic HAp absorption bands, the composite hydrogels possess absorption bands specific for the gelatine matrix: amino (N–H) band at wave number range of 3270 – 3370 cm^{-1}, absorption band associated with C–H stretching at 2947 cm^{-1}, and characteristic amide bands at 1637 cm^{-1} (–C=O) and 1540 cm^{-1} (N–H). Absorption band at 1450 cm^{-1} corresponds to carbonyl bond of ionised $–COO^-$ group[13]. Mentioned absorption bands of gelatine were also found in the composite hydrogels with a slight shift of the C=O asymmetric stretching vibration from 1637 to 1633 cm^{-1}, and the carboxylic group from 1452 to 1448 cm^{-1} observed in the composite with the highest HAp content. This could be a result of specific complexation reactions involving calcium ions from apatite and carboxylic ($–COO^-$)/carbonyl group of gelatine. The complexation of calcium and carboxyl groups during *in situ* apatite formation has been reported in pectin matrices as a possible route for nucleation of hydroxyapatite crystals[14]. As a result, nanostructured materials with good crystal dispersion can be formed.

The mineralogical composition of composite systems and control materials was studied by X-ray diffraction analysis and identified using ICDD (International Centre for Diffraction Data) card catalogue. The XRD patterns of investigated materials

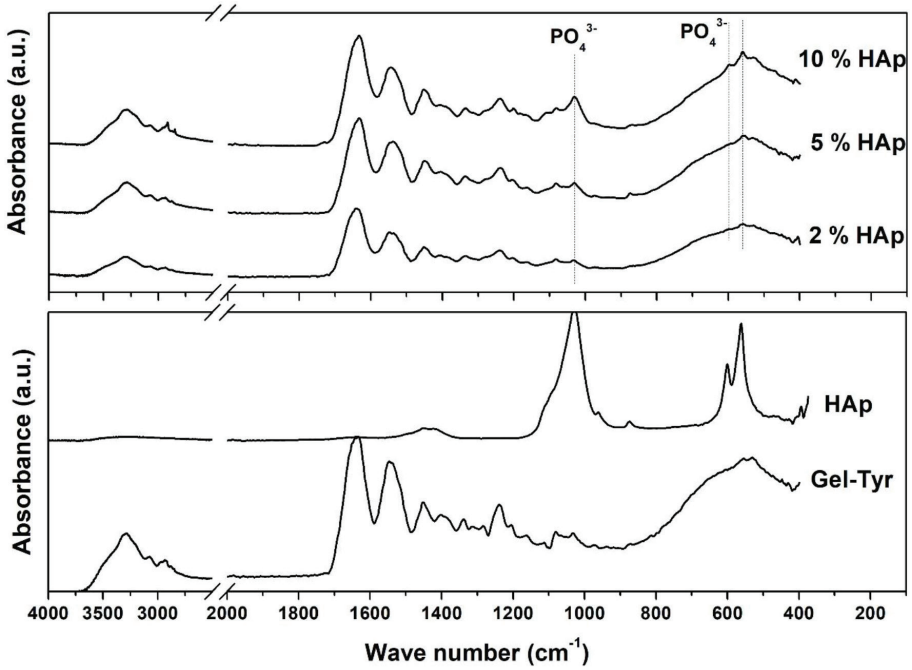

Fig. 2 – *FTIR spectra of gelatine-tyramine (Gel-Tyr), hydroxyapatite (HAp) and Gel-Tyr/HAp composites with different HAp content (2 % HAp, 5 % HAp, and 10 % HAp)*

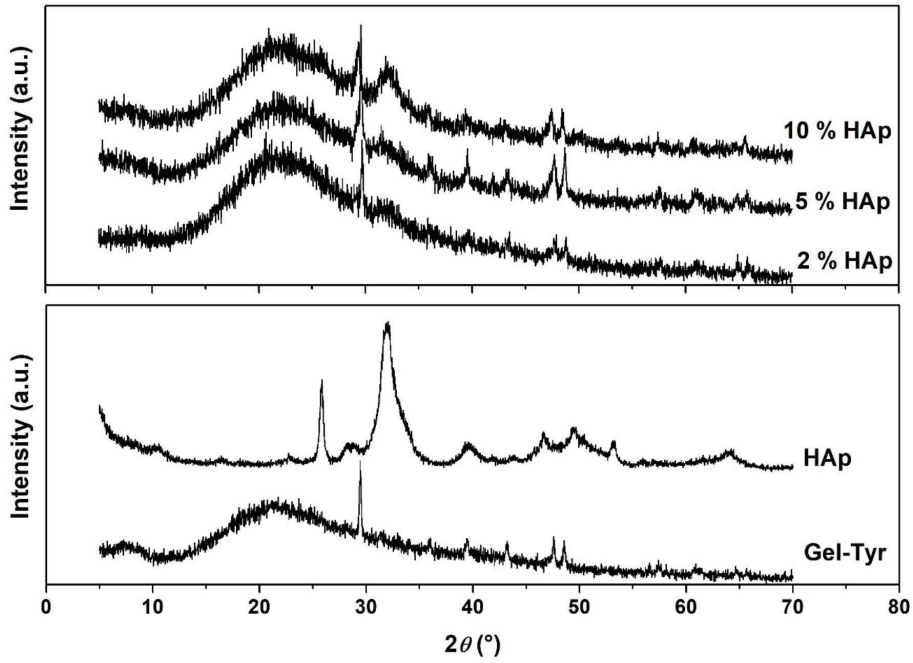

Fig. 3 – *Diffraction patterns of gelatine (Gel-Tyr), hydroxyapatite (HAp) and Gel-Tyr/HAp composites with different HAp content (2 % HAp, 5 % HAp, and 10 % HAp)*

are given in Fig. 3. According to the standard card (ICDD 9-432), control material shows hydroxyapatite as only inorganic phase indicated by diffraction maxima at 2θ = 25.9° (002), 31.9° (211), 39.7° (310), 46.6° (222), 49.4° (213) and 53.4° (004). The broadening of diffraction maxima of HAp patterns suggests low crystallinity, which plays an important role during bioresorption processes. On the other hand, amorphous structure of gelatine-tyramine was observed by broad diffraction maxima at 2θ = 7.2, 8.5 and 21.2°. These characteristic diffraction maxima are usually assigned to the triple-helical crystalline structure in collagen and gelatin[15].

XRD diffraction patterns of *in situ* formed composite hydrogel show superposition of diffraction peaks corresponding to hydroxyapatite and gelatine-tyramine. The apatite phase is indicated by the strongest diffraction maximum at $2\theta = 31.9°$, which is clearly observable in Gel-Tyr/HAp with 10 % of hydroxyapatite. A broader maximum of hydroxyapatite within gelatine indicates low crystallinity of *in situ* precipitated inorganic phase, which could be a result of specific complexation, i.e., nucleation by the carboxylic/carbonyl group. Smaller hydroxyapatite crystals can impact the protein adsorption onto artificial material, which finally dictates cell adhesion during *in vitro* and *in vivo* tissue formation. Besides, the crystalline size can also impact the rate of dissolution and bioresorption of the material. Having smaller HAp crystals, Gel-Tyr/HAp hydrogels could positively influence the cell attachment. The lower intensity of $2\theta = 31.9°$ in 2 % and 5 % of HAp in composite hydrogels could be due to lower apatite content.

Morphology of hydrogels

In order to observe the morphology and distribution of HAp within the gelatine, the cross-section of lyophilised hydrogels was imaged by SEM. The micrographs of cross-section of pure gelatine (Gel-Tyr) and different composite hydrogels are depicted in Fig. 4.

All hydrogels exhibit a layered structure without defined pores size or shape which results from the formation of water crystals during freezing. Pure gelatine-tyramine is characterised by smooth surface, while the addition of hydroxyapatite resulted in smaller or larger particles or crystals agglomeration. The lowest amount of HAp (2 % HAp) shows small agglomerates, consisting of plate-like crystals (indicated with red arrows), concentrated on some parts of the gelatine matrix. The increase in apatite content favours the formation of larger plate-like agglomerates which are homogeneously distributed throughout the gelatine matrix (5 % HAp) with respect to other composites. The highest amount of HAp (10 % HAp) led to the formation of characteristic cauliflower morphology consisting of petal-like crystal agglomerates emerging from the gelatine and concentrated on the edges of 'polymer sheets'. Dispersion of hydroxyapatite crystals within gelatine matrix depends on the quantity of calcium and phosphate precursors, stirring speed, pH of reaction mixture, physicochemical properties of polymer solution, etc.[16] The viscosity of gelatine solution is very low; therefore, it was difficult to obtain stable gelatine/HAp composites at higher HAp quantity. Hydroxyapatite crystals are of nanometric sizes and tend to agglomerate. At physiological pH, as it was for reaction mixture, crystals form specific petal-like or cauliflower structure, i.e., agglomerates. The larger agglomerates with size of approximately 10 μm were formed as a result of the crystal growth provided by the higher concentration of phosphate and calcium ions. Such morphology of *in situ* synthesized hydroxyapatite has been previously reported in chitosan-HAp based systems[17]. However, the application of injectable hydrogels considers utilization of small-gauge needles. The smaller inorganic particles guarantee the efficient injection of such hydrogel[18]. Comparing the investigated composites, 5 % of HAp seems to have better distribution of HAp agglomerates through gelatine matrix, as seen from SEM micrographs.

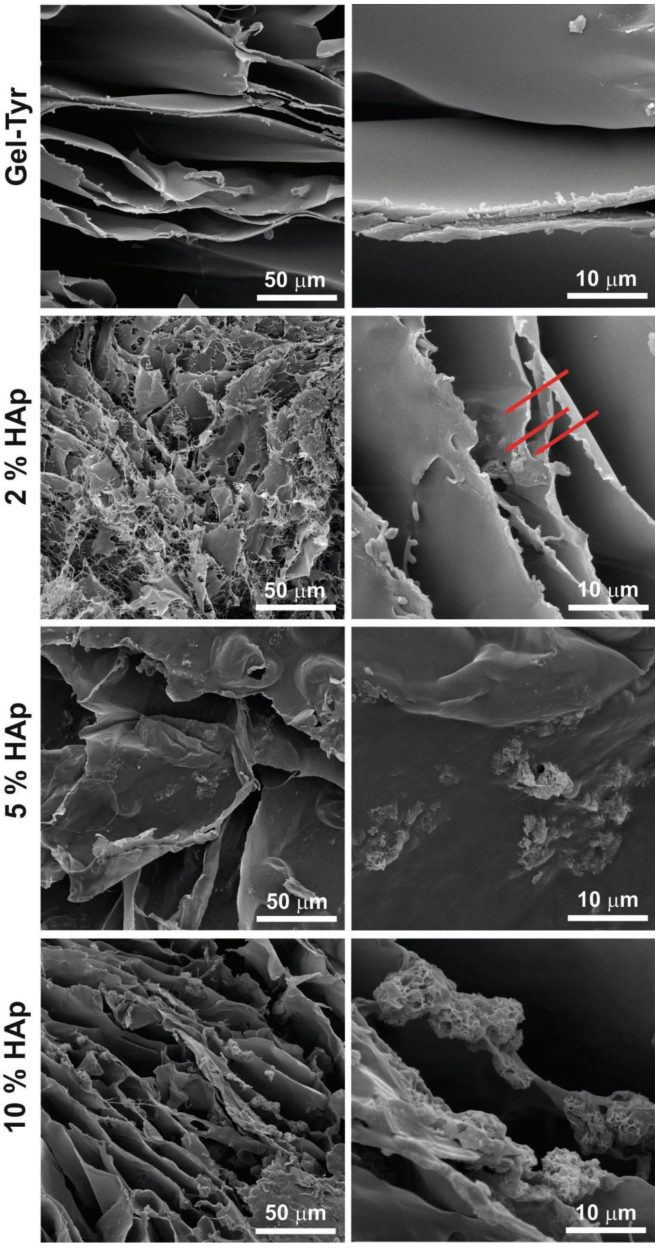

Fig. 4 – *SEM micrographs of gelatine (Gel-Tyr) and Gel-Tyr/HAp composites with different HAp content (2 % HAp, 5 % HAp, and 10 % HAp)*

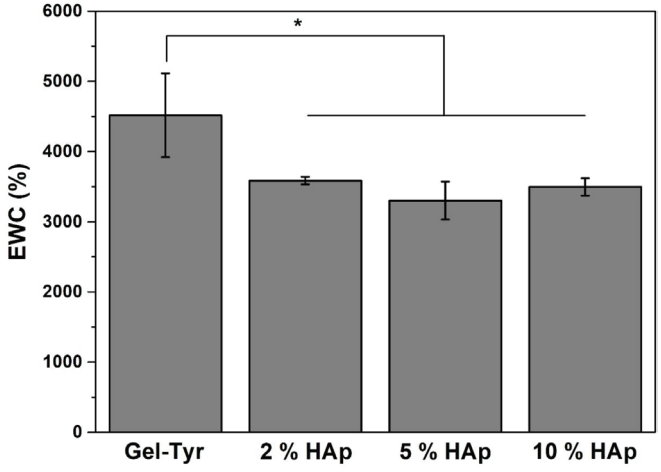

Fig. 5 – *Equilibrium water content (EWC) of gelatine (Gel-Tyr) and Gel-Tyr/HAp composites with different HAp content (2 % HAp, 5 % HAp, and 10 % HAp). Significant difference between two groups is denoted by asterisk (*).*

Swelling ability

The ability of the hydrogel to absorb water was proved to have a great impact on the mechanical stability under load and diffusion of cell nutrient during *in vitro* culture. Swelling behaviour of the hydrogels is closely related to its porosity and crosslinking degree. The swelling capacity decreases with increasing crosslink density[19] and can be altered by the incorporation of different inorganic or polymer particles with different hydrophilicity.

All hydrogels show high swelling ability with equilibrium water content higher than 3000 % (Fig. 5). The largest water uptake is noted for pure gelatine-tyramine (Gel-Tyr) hydrogel with value of ~4500 %. Although hydroxyapatite favours water adsorption, swelling ratio was lowered by the incorporation of HAp down to approximately 3500 % for all Gel-Tyr/HAp hydrogels. This could be a consequence of hindered carbonyl/carboxylic groups of gelatine chains by *in situ* formed hydroxyapatite particles, thus affecting the hydrogel's hydrophilicity.

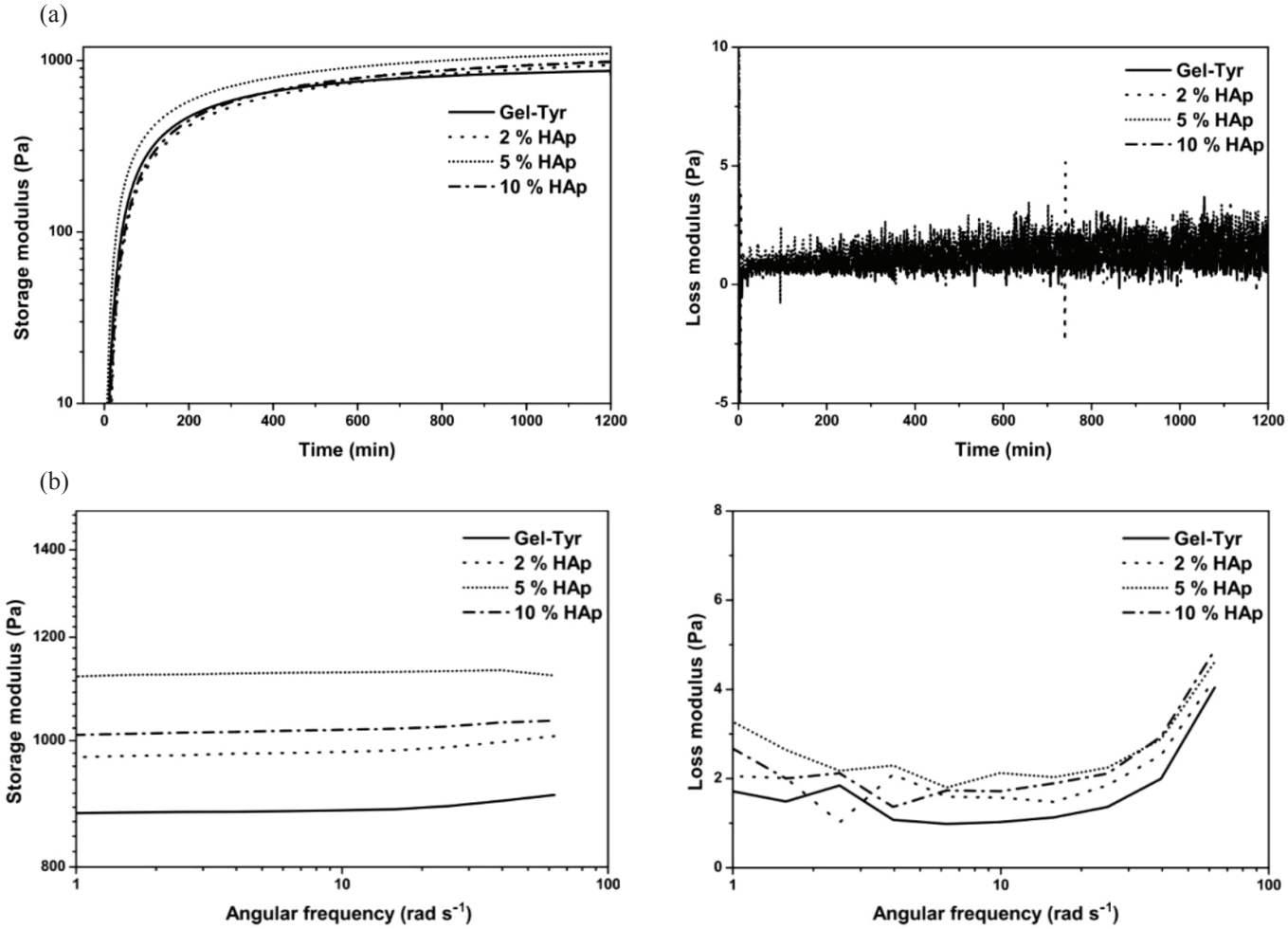

Fig. 6 – *Rheological properties of gelatine (Gel-Tyr) and Gel-Tyr/HAp composites with different HAp content (2 % HAp, 5 % HAp, and 10 % HAp): a) gel formation was monitored by time sweep evaluating the storage and loss moduli as functions of time at 1 % strain; b) frequency sweep data at a constant strain of 1 %. Figure represents curves obtained from average data of three individual samples. Relative standard deviations of storage modulus are not included for the sake of clarity.*

Table 1 – *Gelation time, storage, and loss modulus of different hydrogels evaluated at an angular frequency of 1.26 rad s^{-1} using 1 % of strain at 25 °C. The values represent the average of three individual experiments.*

Hydrogel	Gelation time (s)	Shear storage modulus (Pa)	Shear loss modulus (Pa)	tan δ
Gel-Tyr	212.4 ± 2.5	822.7 ± 32.6	1.2 ± 0.0	$1.5 \cdot 10^{-3}$
2 % HAp	269.8 ± 32.9	942.1 ± 80.3	2.0 ± 0.9	$2.1 \cdot 10^{-3}$
5 % HAp	209.3 ± 0.4	1098.6 ± 54.0	2.5 ± 0.5	$2.3 \cdot 10^{-3}$
10 % HAp	272.6 ± 13.7	984.3 ± 42.5	0.7 ± 0.2	$7.1 \cdot 10^{-4}$

Rheological behaviour of hydrogels

The shear strength of crosslinked hydrogels was evaluated within linear viscoelastic range to ensure their stability[20]. The strain amplitude test, where shear storage and loss modulus are independent of the applied shear stress, showed gel stability up to 15 % strain (data not shown). Subsequent rheological measurements (Fig. 6) were obtained at 1 % strain to ensure linear viscoelastic region free of noise interference.

The gelation time of the injectable hydrogels was evaluated by the time sweep test at 25 °C. The time of crosslink of Gel-Tyr and Gel-Tyr/HAp was determined as a cross-section where interpolation of initial and ending regions of storage modulus–time curve meet[21]. The enzymatically-crosslinked hydrogels are characterised by their fast gelation up to a few minutes. All hydrogels were almost completely crosslinked between 3.3 and 4.5 min (Table 1), after which storage modulus rose slightly (Fig. 6a). After 1200 s of sweep test, the storage modulus reached a plateau. The hydrogel with 5 % HAp seems to have minimum impact on gelation rate regarding pure Gel-Tyr hydrogel. Nonetheless, all prepared composite hydrogels gelate quickly, which is eligible in clinical practice.

The frequency sweep experiments (Fig. 6b) were carried out to investigate the gel properties of the cured systems, namely, the stability of three-dimensional crosslinked networks. No change was found in the storage modulus until 10 rad s^{-1}; however, it increased after 10 – 20 rad s^{-1} depending on the hydrogel's composition. At 1 % strain, the shear strength of hydrogels increased almost linearly with higher HAp content (up to 5 % of HAp) indicating possible reinforcement of the gelatine-tyramine matrix. An unexpected behaviour was observed for the hydrogel with 10 % of HAp that has a lower storage modulus than the hydrogel with 5 % HAp, and of the order of magnitude of the 2 % HAp hydrogel (Table 1). Hydrogel mechanical properties depend on multiple parameters, such as porosity, swelling ability, rigidity of molecular chain, and additional fillers. Since there was no significant difference in the swelling capacity of the different Gel-Tyr/HAp hydrogels, the decrease in the mechanical properties of the 10 % HAp composite can be attributed to the non-uniform concentration of larger HAp agglomerates (as shown in Fig. 4). The limited reinforcement ability of HAp was previously reported on dry polymer/HAp systems as well as for composite hydrogels where increase in HAp amount weakened the mechanical properties[22–24]. The evaluation of compressive and tensile strength of chitosan/HAp porous structures in wet state[24] showed the initial reinforcement of composite when incorporated amount of HAp was low, followed by a decrease in strength at higher HAp content. Similar behaviour was reported on injectable n-HAp/(PEG-PCL-PEG) hydrogel, where rheological evaluation showed decrease in storage modulus when HAp was loaded above 20 %[25]. The same trend was observed in this study; however, at lower range of HAp content than found in the literature. As seen from morphology imaging, 5 % of HAp could be the critical quantity for better dispersion of smaller crystal agglomerates that positively influenced mechanical behaviour of the composite hydrogel.

Cytotoxicity of hydrogels

The cytotoxicity assay is an easy test to determine whether investigated biomaterials are toxic for living cells. High cell viability (over 60 %) indicates non-cytotoxicity of the materials, i.e., good cell spreading and survival. Fig. 7 depicts high cell viability of Hek293 cells cultured in the materials' extracts with respect to the non-treated cells. Moreover, cell proliferation continued for 3 days of culture, indicating favourable composition of composite hydrogel with 5 % of HAp.

Conclusion

Gelatine and hydroxyapatite are frequently used components for development of potential bone tissue grafts due to their biocompatibility, biodegradation, and osteoinductivity. In this work, injectable composite hydrogels based on tyramine-modified gelatine and *in situ* synthesized apatite have been prepared and characterised. *In situ* synthesis of hydroxyapatite led to the formation of different sizes of particles consisting of nano-scale crystals distrib-

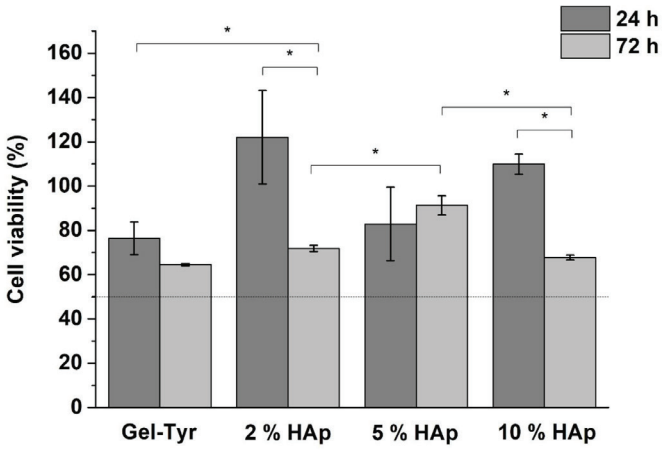

Fig. 7 – *Cell viability of Hek293 cells cultured with material's supernatant after 24 and 72 h of culture. The significant difference between two groups is denoted by asterisk (*) with p < 0.05. 50 % of cell viability is defined as a lower limit of non-cytotoxicity.*

uted through gelatine matrix. Depending on the HAp quantity, mechanical properties of hydrogel could be improved in terms of shear storage modulus. The composite consisting of 5 % HAp possessed the highest increase in shear modulus without significant influence on the gelation time, which could be due to better distribution of smaller HAp agglomerates with respect to other systems. Additionally, the lack of toxicity of prepared injectable hydrogels shows good potential for further investigation of biological properties in terms of their bioactivity and osteoinductivity.

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