ENZYMATIC PREPARATION OF MODULATED–BIODEGRADABLE HYDROGEL NANOCOMPOSITES BASED CHITOSAN/GELATIN AND BIPHASIC CALCIUM PHOSPHATE NANOPARTICLES

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ABSTRACT

In the study, injectable chitosan–4 hydroxyphenylacectamide acid (CHPA) and gelatin–tyramine (GTA)–based hydrogels were enzymatically prepared, in which could encapsulate biphasic calcium phosphate nanoparticles (BCP NPs) for enhancing bone regeneration. The *in situ* formation of hydrogel composite was varied from 35 to 80 seconds depending on concentration of H_2O_2. Collagenase–mediated biodegradation of the hydrogel composite could be modulated from 3 days to over one month depending on amount of the formulated CHPA. Live/dead cell viability assay indicated that the hydrogel composite enhanced bone marrow mesenchymal stem cells (MSCs). The obtained results show a great potential of the hydrogel composites for bone regeneration due to its adjustable biodegradation, biocompatibility and enhancement in new bone formation.

Keywords: chitosan, gelatin, horseradish peroxidase (HRP), hydrogel, collagenase.

1. INTRODUCTION

Recently, biological hydrogels have played an important role in the advanced biomaterials for tissue regeneration and drug delivery systems. Several kinds of the injectable hydrogels performed an effective encapsulation of drugs/cells and convenience for applying the minimally invasive implant surgery [1]. The hydrogels play a role as an artificial extracellular matrix (ECM) inside body for cell migration and proliferation allowing transportation of nutrients

substances and by–products from cell metabolism [2]. These scaffolds could also be encapsulated with peptide, growth factor and etc. for enhancing cell attachment and proliferation. These hydrogels have been prepared via physical, chemical or enzymatic reactions. For enzyme–fabricated hydrogels, they used some specific cross–linked reactions which avoids by–products in network formation. Therefore, the approach performs characteristics more closely integrating artificial materials with biological entities [3].In this study, we introduce an injectable hydrogel composites from CHPA and GTA in the presence of the BCP NPs, HRP enzyme and H_2O_2. It is well–known that chitosan and gelatin are biocompatible materials. Chitosan–based gels possess several beneficial properties for tissue regeneration such as tissue adhesion, anti–infective activity and enhancement of cell attachment [4]. Gelatin has been used for biomedical applications due to its high biocompatibility, fast biodegradability, enhancement of cell attachment and proliferation [5, 6]. Gelatin–based hydrogels were bio property; through, the hydrogels are quickly degraded by collagenase within 3–4 days [7]. Using different weight ratios of chitosan and gelatin, the hydrogel could adjust its biodegradation rate in the presence of collagenase. The hydrogel composite could be potential in different tissue regenerations as well as reducing limitation of gelatin–based materials in biomedical applications.

2. EXPERIMENTAL

2.1. Materials

Chitosan (100 kD, 75–85% deacetylation), gelatin from porcine skin (Bloom 300, type A, MW 100kD), 4–hydroxylphenylacetic acid (HPA) and tyramine (TA), 1–ethyl–3–(3–dimethylaminopropyl) carbodiimide (EDC), which were obtained from Acros Organics. Collagenase and HRP enzyme (type VI) were purchased from Sigma–Aldrich.

2.2. Preparationof polymers

2.2.1. Preparation of 4–hydroxyphenylacetic acid conjugated chitosan (CHPA)

In a flask, chitosan (1g) was dissolved in the solution of 40 mL DI water and HCl 1.0M solution (0.50 mL). 4–Hydroxyphenylacetic acid (0.45g, 2.9 mmol) was added into the flask. pH of solution was adjusted to 5 and then EDC (0.90 g, 4.7 mmol) added to the reaction under stirring for 24 h. The solution was dialyzed against deionized water using membrane dialysis (molecular weight cut–off (MWCO) 6000–8000) for 3 days. Subsequently, the modified chitosan solution was lyophilized to obtain CHPA as shown in Figure 1 (the yield was 0.9g). ^{1}H NMR (D_2O)/ppm: δ 2.05 (s, –$COCH_3$, chitosan); δ 3.22 (m, –C_2(H), chitosan); δ 3.43–3.92 (m, $C_{3+4+5+6}$, chitosan); δ 2.89 (d, –CH_2–, HPA); δ 6.89 và 7.22 (d, –CH=CH–, HPA).

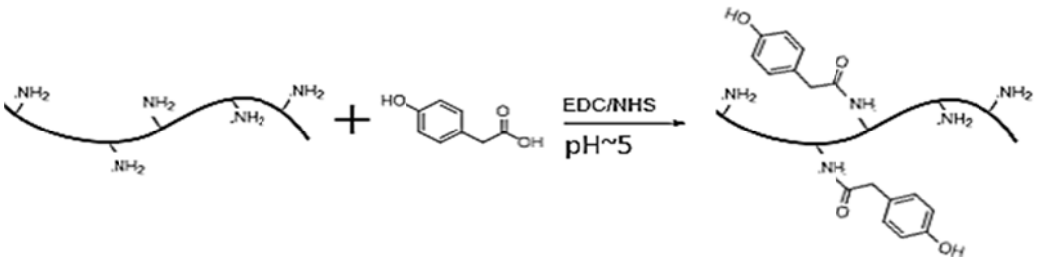

Figure 1. Synthetic scheme of CHPA.

2.2.2. Preparation of tyramine conjugated gelatin (GTA)

Gelatin skin (2g) and TA (1.00 g, 7.3 mmol) were dissolved in DI water (30 mL). The pH of the mixture was adjusted to 6 following addition of EDC (0.50 g, 2.5 mmol) under stirring for 24 h. Then, the solution was dialyzed against deionized water using membrane dialysis (MWCO6000–8000) for 3 days. Subsequently, the dialyzed solution was lyophilized to obtain GTA as shown in Figure 2. Theyield was 1.80 g. ^{1}H NMR (D_2O)/ppm: δ 6.75 and 7.11 (d,–CH=CH– of TA), δ 2.65 and 2.88 (m, $–CH_2CH_2–$, TA).

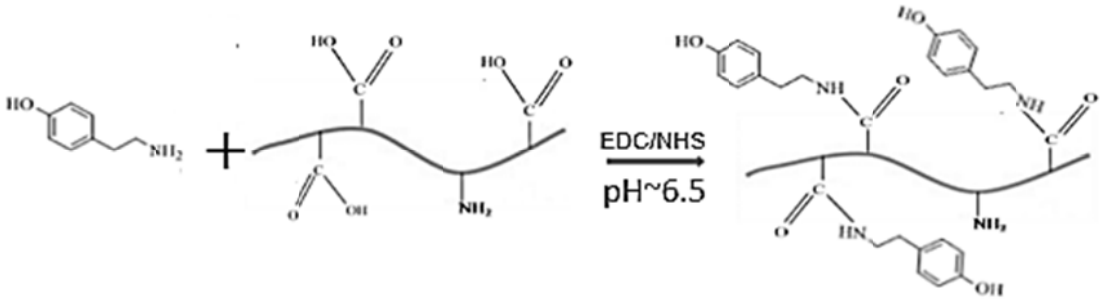

Figure 2. Synthetic scheme of GTA.

2.2.3. Preparation of BCP

BCP were synthesized using an ultrasonic assisted process. The calcium chloride reacted to tricalcium phosphate salts with molar ratio of Ca/P = 1.57 for 12 h at 50 °C under controlled pH 7 to obtain a white suspension. The pH solution was maintained by adding of sodium hydroxide solution. The precipitate was washed with DI water and dried in an oven at 70 °C. Finally, the calcination was carried out at 750 °C.

2.3. Preparation of gelatin and chitosan–based hydrogels

GTA (40 mg) was dissolved in DI water (300 µL) and separated into two vials equally. Then, enzyme HRP (30 µL of 0.07 mg/mL) and H_2O_2 (30 µL of 0.03–0.07 wt/vol%) were added into each tube. GTA hydrogel was formed by mixing the solution of 10 wt/wt% polymer. CHPA hydrogel was prepared by a same above process. HRP (30µL of 0.05 mg/mL) and H_2O_2 (30 µL of 0.05–0.2 wt/vol%) were added into each tube. The final concentration of the polymer solution was 2 wt/wt%. The gelation time was determined by using the vial tilting method.

2.4. Preparation of chitosan/gelatin–based hydrogels, hydrogel composites

Precursor polymer solutions were prepared in four vials. In each vial A and B, GTA (20 mg) were dissolved completely in DI water (150 µL). In vial C and D, CHPA (10 mg) disslolved in DI water (160 µL). 30 µl HRP (0.07 mg/mL) was added into A, C and 30 µL H_2O_2 (0.05–0.2 wt/vol%) was added into B, D. A was mixed with C, B was mix with D. Finally, two polymer solutions containing HRP and H_2O_2 were interfused together to create *in situ* formation GTA or CHPA hydrogels at 8 wt/wt% of the polymer concentration. The hydrogel composites containing BCP nanoparticles (10 wt/wt%) was prepared by the same manner. The gelation time of the samples were studied based on variation of the concentration of H_2O_2 from 0.05, 0.07, 0.1, 0.2wt/vol%.

2.5. *In vitro* biodegradation study

The *in vitro* biodegradation of hydrogel, hydrogel composites were studied immersing hydrogels and hydrogel composites in PBS solution with the presence of collagenase (0.2 U/mL) at 37 °C and then monitored their weight–losses following different incubation times. The enzymes were prepared in a PBS 0.01 M, pH = 7.4 solution. The samples with different mass ratios were accurately weighted before immersing in 1 mL of enzymatic solution. At the predetermined intervals, the samples were removed from the incubation medium. Then the weight of degraded hydrogels, hydrogel composites (W_t) was measured to determine the weight of the remaining the samples. Degradation rate (rate of weight loss %) $= \frac{Wi-Wt}{Wi}.100$ % W_i and W_t are initial weights of hydrogels or hydrogel composites and degraded hydrogels or hydrogel composites, respectively.

2.6. Characterization

The structures of CHPA and GTA were determined by using NMR at Institute of Chemical–VAST (Varian, 400 MHz, U.S.A) at 37 °C and an UV–Vis spectrophotometer (JASCO V–570, Japan). Morphology of BCP was determined by using Field–emission scanning electron microscope (FESEM) JSM–635F, JEOL. The measurement was conducted at Institute of Chemical Technology–VAST. The phase analysis of the BCP NPs was identified using an X–ray diffractometer (XRD, D8/Advance, Bruker, UK) with CuKα, (λ = 1.5406 Å) at Institute of Applied Materials Science–VAST.

3. RESULTS AND DISCUSSION

3.1. Characterizations of polymers

Recent years, the HPR enzymatically cross–linked reactions have played a crucial role in preparation of several polysacharide–based hydrogels [3]. Conjugation of HPA on chitosan formed a phenolic derivative enable to exploit for enzyme–mediated cross–linking reaction. The HPA–conjugated chitosan was confirmed from resonance signals of aromatic protons of HPA at 6.89 and 7.22 ppm (Figure 3, top). The signals at 2.89 ppm were assigned to methylene protons of HPA. Overlapped, broad resonance signals of D–glucosamine of chitosan were observed in the interval 3–4 ppm.

GTA can be also synthesized by the coupling reaction using EDC. Tyramine grafted gelatin was determined by the resonance signals (2.65 ppm and 2.88 ppm) of the methylene protons of tyramine. Peaks of aromatic protons of tyramine appeared at 6.75 and 7.11ppm. Some signals of amino acids in ^{1}H NMR spectrum were shown (Figure 3, bottom): δ 4.55 and 4.68 (–CH_2–, proline); 4.27 (methine proton of hydroxyproline); 3.88 (–CH_2–, alanine); 1.34 (–CH_3, alanine); 3.57 (–CH_2–, glycine); 2.23 (–CH_2–, glutamic acid); 1.60(–CH_2–, arginine); 3.14, 7.23 and 7.29 methine proton of phenylalanine).

These ^{1}H NMR results could confirm the successful preparation of two phenolic precursors for fabricating the hydrogels.

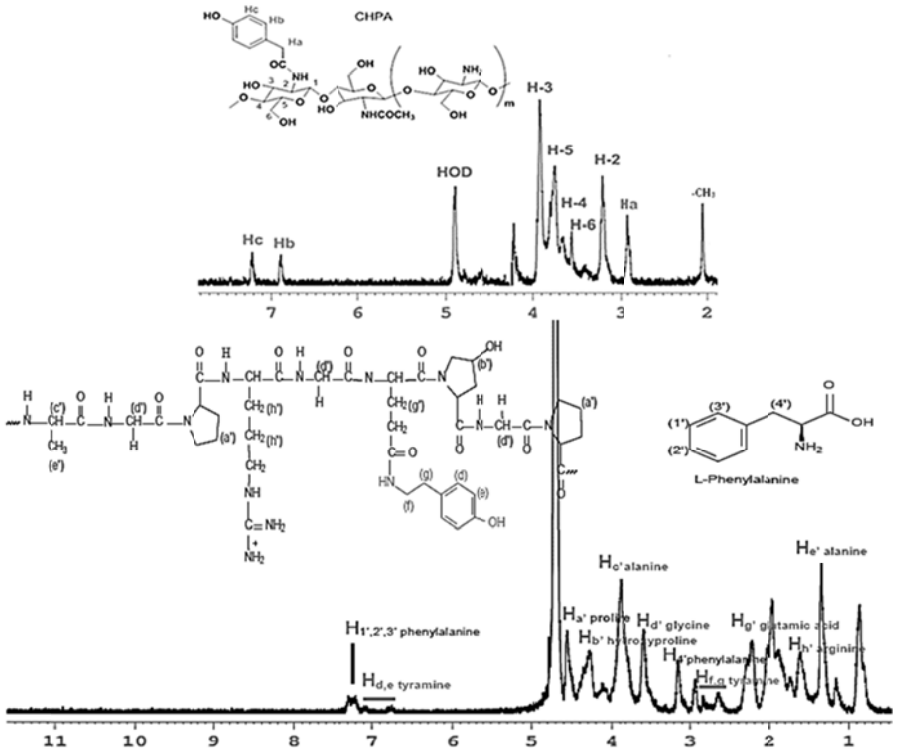

Figure 3. ^{1}H NMR spectrum of chitosan 4–hydroxyphenylacetic acid (CHPA, top) and gelatin–tyramine (GTA, bottom) in D_2O.

3.2. Characterizations of BCP

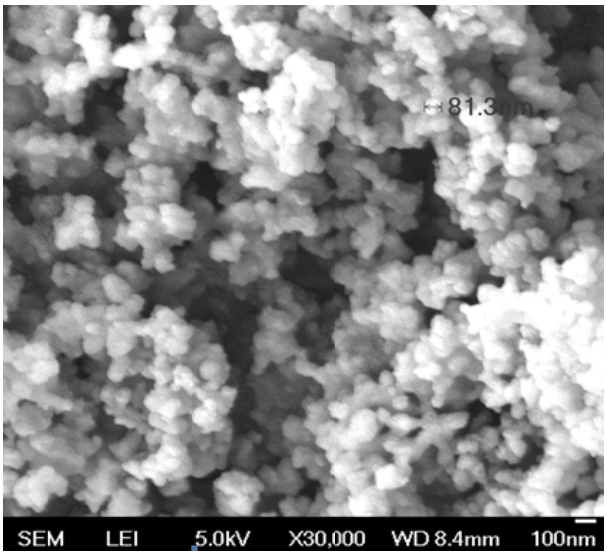

Figure 4. SEM image of the BCP nanoparticles.

Figure 4 shows the SEM images of BCP nano powders which were synthesized using ultrasound irradiation. The synthesized BCP powders had a spherical shape and diameter ranging from 60 to 100 nm. The ultrasound promotes chemical reactions and physical effects; ultrasonic cavitation improves the material transfer at particle surfaces. Therefore, use of the ultrasound–assisted method can synthesize smaller particle size and higher uniformity due to good mixing of the precursors.

3.3. Characterizations of hydrogels, hydrogel composites and gelation time

As mentioned, using phenolic moieties containing polymers and HRP/H_2O_2–mediated coupling reaction is interesting approach to prepare hydrogels. The gelation of the polymer solutions occurs by coupling of phenol moieties [3]. In case, HRP promotes the degradation of H_2O_2 into radicals which initiate the formation of gel. Gelation formed within a few period time and formed a strong and highly elastic gel (Figure 5).

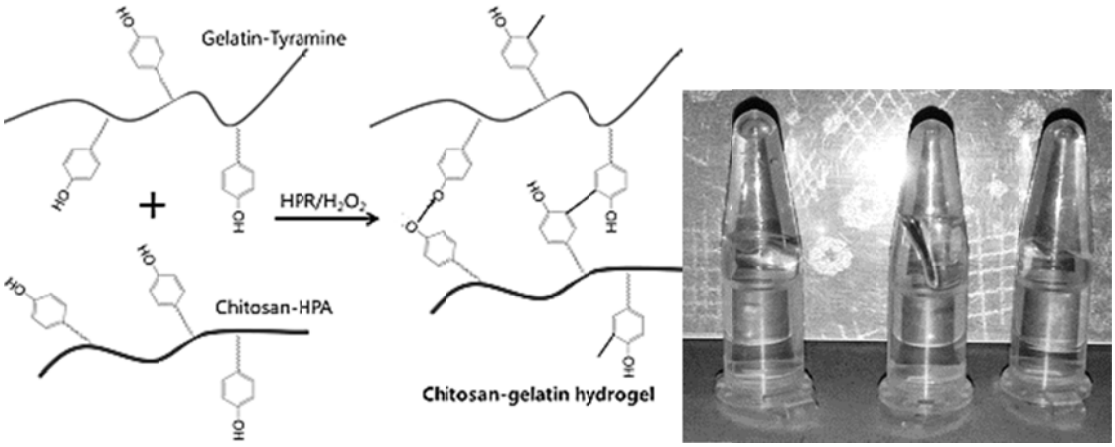

Figure 5. Formation of gelatin and chitosan–based hydrogels.

Figure 6a, bindicate that the lowest gelation time was obtained at 0.07 wt% concentration of H_2O_2 and HRP (0.05 mg/mL) in 12 s for CHPA hydrogel and in 50 s for GTA hydrogel at 0.05 wt% concentration of H_2O_2 and 0.07 mg/mL concentration of HRP. This could result in the numbers of phenolic groups coupled to GTA were less than to CHPA. In cases of CHPA gel and GTA gel, an increment of H_2O_2 concentration at the fixed HRP could lead to extending the gelation time because more phenolic radicals were produced in their polymer solutions. Figure 6cindicated that CHPA–GTA hydrogels and hydrogel composites could be formed below one and half minutes depending on amount of H_2O_2. It is a lightly difference in gelation time of the hydrogel and hydrogel composite that contributes from presence of BCP NPs. Functional NH_2, OH, COOH groups of gelatin and amine groups chitosan link with OH groups of HAp in BCP resulting in increasing cross–linking density of hydrogel composite so gelation time of hydrogel composites decreased. In the study, it is important to use an amount of hydrogen peroxide in a cell–favorable range that doesn't induce cell apoptosis. So molar ratio of H_2O_2 and phenol groups should be matched each other. In the fact, concentration of H_2O_2 could be significantly decreased in the process of the hydrogel formation due to oxidation of phenol moieties.

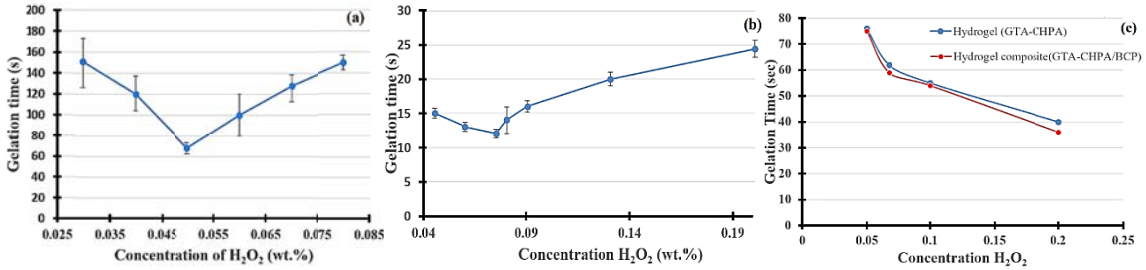

Figure 6. Effect of H_2O_2 concentration on gelation time of hydrogel with a) 0.07 mg/mL HRP for GTA gel, b) 0.05 mg/mL HRP for CHPA gel and c) 0.07 mg/mL HRP for CHPA–GTA hydrogel composite.

3.4. *In vitro* biodegradation study

The proteolytically degradable property of the artificial matrix plays a crucial role in the tissue regeneration. Figure7 shows the collagenase–mediated degradation behavior of the materials with different mass ratios of formulated chitosan (C) and gelatin (G). The degradation rate decreased following the decrease in amount of gelatin in hydrogel. For instance, hydrogels at a mass ratio of 0C:10G were completely degraded after 42 hours and degraded after 90 hours for the mass ratio of 0.5C:10G. In contrast, chitosan/gelatin–based hydrogels at the mass ratios of 1C:10G; 1C:5G; 1C:2.5G were not utterly degraded within 762 hours. There was a prolonged degradation rate of all hydrogel composite sample in comparison with hydrogels. This could be explained that presence of calcium and phosphate ions released from BCP NPs participating to cross–linking reaction with amine and carboxylate groups in polymers resulting in increasing cross–linking density.

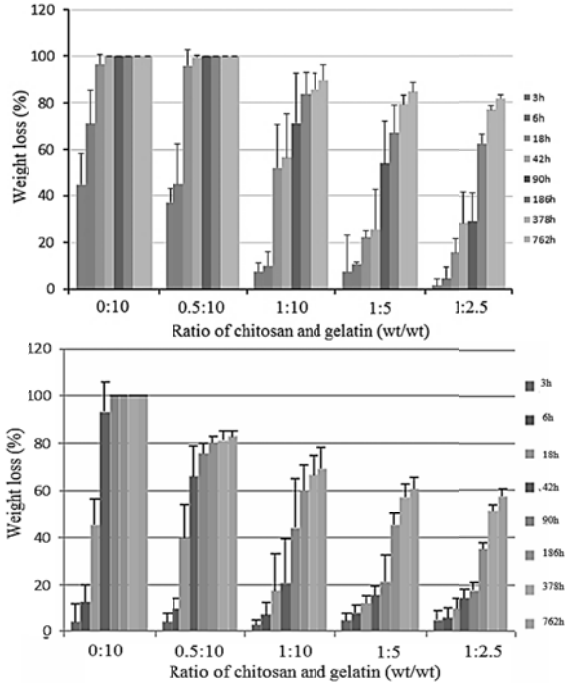

Figure 7b. In vitro biodegradation rate of hydrogels GTA–CHPA (top) and hydrogel composite GTA–CHPA (bottom) in presence of collagenase enzyme.

This result may be explained by the fact that gelatin–based materials have a fast degradable profile. Incorporating with chitosan, the hydrogel could adjust its biodegradation rate in the presence of collagenase. The preliminarily obtained results are significant because the hydrogel composites could be selected to implant into human body to regenerate every specific tissue.

4. CONCLUSIONS

In situ forming hydrogel composites consisted of tyramine conjugated gelatin, 4–hydroxyphenylacetic acid conjugated chitosan and BCP were successfully prepared via horseradish peroxidase mediated reaction in the presence of hydrogen peroxide. With a rapid gelation time at the physiological condition and controllable biodegradation rate, the hydrogel composites will be significant to apply in regenerative medicine.

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REFERENCES

1. Schmaljohann D. – Thermo– and pH–responsive polymers in drug delivery, Advanced Drug Delivery Reviews **58** (15) (2016) 1655–1670.
2. John A., Sharon K., Luke M., Declan M. D, Eoin S, Daniel B, Clement L. H. – Hydrogel/bioactive glass composites for bone regeneration applications: synthesis and characterization, Materials Science and Engineering C **33** (2013) 4203–4212.
3. Kurisawa M., Chung J. E., YangY. Y., Gao S. J., Uyama H. – Injectable biodegradable hydrogels composed of hyaluronic acid–tyramine conjugates for drug delivery and tissue engineering,Chemical Communications**34** (2005) 4312–4314.
4. Kim I. Y., Seo S. J., Moon H. S., Yoo M. K., Park I. Y., Kim B. C., Cho C. S. – Chitosan and its derivatives for tissue engineering applications,Biotechnology Advances **26** (2008) 1–21.
5. Ueno H., Nakamura F., Murakami M., Okumura M., Kadosawa T., Fujinag T. – Evaluation effects of chitosan for the extracellular matrix production by fibroblasts and the growth factors production by macrophage, Biomaterials**22** (2001) 2125–2130.
6. Dreesmann L., Ahlers M., Schlosshauer B. – The pro–angiogenic characteristics of a cross–linked gelatin matrix, Biomaterials**28**(36) (2007)5536–5543.
7. Yunki L., et al. – *In situ* forming gelatin– based tissue adhesives and their phenolic content–driven properties, Journal of Materials Chemistry B **1**(2013) 2407–2414.