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Enzyme-mediated preparation of the gelatin/alginate-based nanocomposite hydrogel

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Abstract

In this study, derivatives of gelatin and alginate were combined with biphasic calcium phosphate (BCP) to produce hydrogel composites (NCgel) which could be ultilized for bone regeneration. The gelatin-tyramine (GEL-TYR) and alginate-tyramine (ALG-TYR) were prepared by the coupling reaction via 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) at pH 6 and these products were identified by ^{1}H-NMR, FTIR. BCP nanoparticles were synthesized by using an ultrasonic associated process with rate Ca/P 1.57 at pH = 7. Then, GEL-TYR, ALG-TYR and BCPs were utilized to produce NCgel via enzyme horseradish peroxidase (HRP)-mediated reaction in the presence of hydrogen peroxide (H_2O_2). The hydrogel and hydrogel composite products were surveyed the gelation time, biodegradation in enzyme collagenase solution after 1524 hours. Our preliminary results demonstrated a great potential of the hydrogel composite for biomedical applications.

Keywords. Alginate, gelatin, BCPs, tyramine, bone regeneration, biodegradation.

1. INTRODUCTION

Hydrogels, which produced by natural polymers, have many biologically beneficial properties, such as high water-swollen, biodegradability and biocompatibility for cell migration and proliferation. Certainly, they will be ideal materials to apply in biomedical field. 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. Recently, there has been an emerging approach use of the NCgel. The nanocomposite material combines the benefit properties of the hydrogel materials and reinforced property of particles [1]. Moreover, some kinds of phosphate nanoparticles could stimulate growth of osteoblast cell and biomineralization of the NCgel that performs a great potential for bone regeneration.

In the present study, according to differently collagenase-induced biodegradation of gelatin and alginate as well as its cytocompatibility, injectable alginate-tyramine and gelatin-tyramine (GTA)-based hydrogels were enzymatically prepared, in which could encapsulate biphasic calcium phosphate nanoparticles (BCP NPs) for enhancing bone regeneration.

2. EXPERIMENTAL

2.1. Materials

Alginate sodium (Sigma Aldrich) Gelatin (Merck), tris($CH_2OH)_3CNH_2$ (Sigma Aldrich), tyramine (TA) (Acros organics), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Acros organics), hydrogen peroxide (Acros organics), horsera dish peroxidase (HRP) (Acros organics), sodium chloride (Scharlau), Calcium chloride ($CaCl_2.2H_2O$) (Merck), sodium hydroxide (Merck), disodiumhydro-phosphate ($Na_2HPO_4.2H_2O$) (Merck), hydrochloric acid (Merck), collagenase (Sigma Aldrich).

2.2. Preparation of polymer

2.2.1. Preparation of gelatin- tyramine (figure 1)

In round bottom flask, 2 g gelatin was dissolved in 30 mL distilled water at 40 °C. After cooling down to room temperature, 1 g tyramine was added and gelatin solution was adjusted to pH 6 with HCl 5%. Then, 250 mg EDC was added and the reaction takes place in 24 hours under stirring. Finally, the solution was dialyzed against deionized water for 3 days and Freeze dried in 24 h. The derivatives of gelatin were identified by ^{1}H-NMR, FTIR.

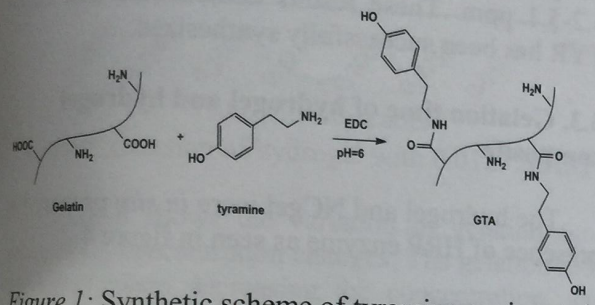

Figure 1: Synthetic scheme of tyramine conjugated gelatin (GEL-TYR)

2.2.2. Preparation of alginate- tyramine

In round bottom flask, 2 g alginate sodium was dissolved in 125 mL distilled water. Tyramine 3 g was added and adjusted to pH = 6 with HCl 5 %. Then, 1.94 g EDC was added and the reaction takes place in 24 hours under stirring. Finally, the solution was dialyzed against deionized water for 3 days and Freeze dried in 24 h. The derivatives of alginate were identified by ^{1}H-NMR, FTIR.

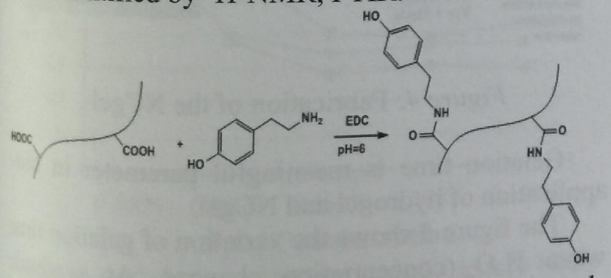

Figure 2: Synthetic scheme of tyramine conjugated alginate (ALG-TY)

2.2.3. Preparation of BCP

BCP nanoparticles were synthesized by the precipitated method combined with ultrasonic, molar ratio Ca/P = 1.57 and pH 7 [2].

$CaCl_2.2H_2O$ (6.93 g) and $Na_2HPO_4.2H_2O$ (5.34 g) were dissolved in 100 mL H_2O, separately. Two solutions were adjusted to pH 7 with 1 M NaOH or HCl 5%. Na_2HPO_4 solution was dropped into $CaCl_2$ solution under sonication. Precipitating process takes place in 12 hours. Then, the product is filtered, dried, calcined at 750 °C for 2 hours and finely ground.

2.3. Preparation of hydrogel and NCgel

Hydrogel: 10 mg polymer (GEL-TY (G) or ALG-TY (A) was dissolved in 200 µL PBS buffer with pH 7.4. The solution was divided into two equal parts. 20 µL of 0.2 % H_2O_2 was added to part 1 and 20 µL of 0.0125 % HRP was added to part 2. After mixing two parts together, hydrogel was formed.

NCgel: 10 mg polymer (GEL-TY or ALG-TY) was dissolved in 200 µL PBS buffer with pH 7.4. BCP particles were dispersed in the solution. Then, the mixture was divided into two equal parts. 20 µL of 0.2 % H_2O_2 was added to part 1 and 20 µL of 0.0125 % HRP was added to part 2. After mixing two parts together, NCgel was formed.

2.4. Biodegradation of hydrogel and NCgel

Hydrogel and NCgel samples were prepared according to the table 1.

Table 1: Synthetic composition of hydrogel and NCgel

A:G ratio	Weight (mg)			V_{PBS} (µl)	V_{H2O2} (µl)
	ALG-TY	GEL-TY	BCP		
1:0	10	-	-	180	20
2:1	6	3	-		
1:1	5	5	-		
1:2	3	6	-		
1:4	2	8	-		
1:0	10	-	10		
2:1	6	3	10		
1:1	5	5	10		
1:2	3	6	10		
1:4	2	8	10		

Hydrogel and NCgel were created after mixing two polymer solutions together. Samples were soaked in collagenase solution (16 mg/1L SBF solution at pH 7.4) at room temperature [3]. The weight change of samples was recorded after 3, 6, 18, 42, 90, 186, 378, 762 and 1524 h soaking.

$$\text{Weight change} = \frac{W_t - W_o}{W_o}.100\% \quad (1)$$

W_o: Initial weight of gel; W_t: weight of gel at time t

2.5. Gelation time of the hydrogel and NCgel

Hydrogel and NCgel samples were prepared

according to method with difference A/G ratios in table 1. The various H_2O_2 concentrations are 0.4, 0.2, 0.175, 0.15, 0.1 and 0.075 % and the HRP concentration is permanent 0.0125 %. Gelation time was determined when the reaction solution transferred from a liquid into a gel (not flow anymore when upside down).

2.6. Characterization

The structures of the synthetic polymers were determined by using NMR at Institute of Chemistry-VAST (Varian, 400 MHz, U.S.A) at 37°C. 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 CuK_α, (λ = 1.5406 Å) at Institute of Applied Materials Science - VAST.

3. RESULTS AND DISCUSSION

3.1. Synthetic gelatin tyramine

Figure 3 shows some typical peaks on ^{1}H-NMR spectra that performs GEL-TYR structure. 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 7.11 ppm. 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).

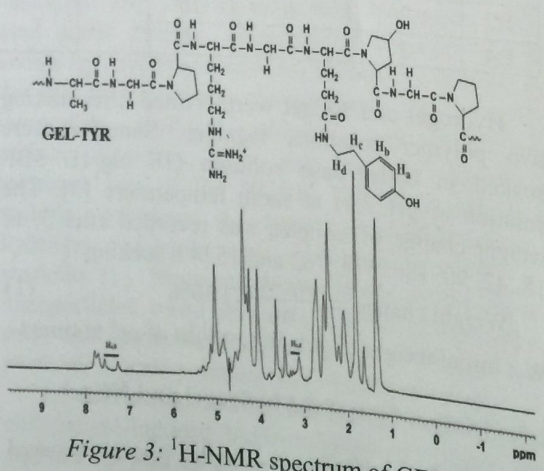

Figure 3: ^{1}H-NMR spectrum of GEL-TY

These ^{1}H-NMR results could confirm the successful preparation of two phenolic precursors for fabricating the hydrogels [2].

3.2. Synthetic alginate tyramine

Result analysis of structure of ALG-TYR is identified by ^{1}H-NMR spectra. Two signal at δ 6.8 and δ 7.2 ppm demonstrate the presence of Ha and Hb (aromatic proton of tyramine). Some peaks assign to polysacride protons of ALG appeared at 3.2-5.1 ppm. These results demonstrate that ALG-TYR has been successfully synthesized.

3.3. Gelation time of hydrogel and hydrogel composite

The hydrogel and NCgel were *in situ* prepared in presence of HRP enzyme as seen in figure 4.

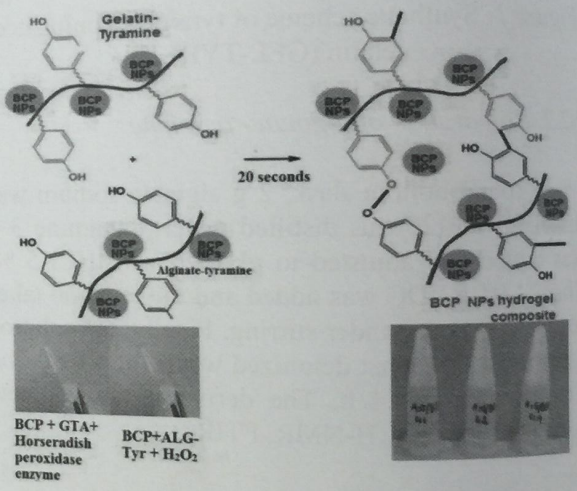

Figure 4: Fabrication of the NCgel

Gelation time is meaningful parameter in bio-application of hydrogel and NCgel.

The figure 5 shows the variation of gelation time when H_2O_2 concentration changes. At synthetic composition of 1A:0G ratio, the gelation time decreases when decrease the H_2O_2 concentration, and the gelation time is constant in the range of 0.1-0.15 % concentration of H_2O_2. Similar to 1A:0G ratio, 2A:1G has the gelation time decreases when decrease the H_2O_2 concentration, and the gelation time isn't change in the range of 0.1-0.2 % concentration. The gelation time is long at high concentration can be explained by enzyme activity is inhibited at high concentration of H_2O_2. If H_2O_2 concentration is lower 0.1 %, gel formation doesn't occur. In 1A:1G ratio, concentration doesn't affect gelation process in range 0.05-0.4 %, but lower

H_2O_2 concentration is unavailable.

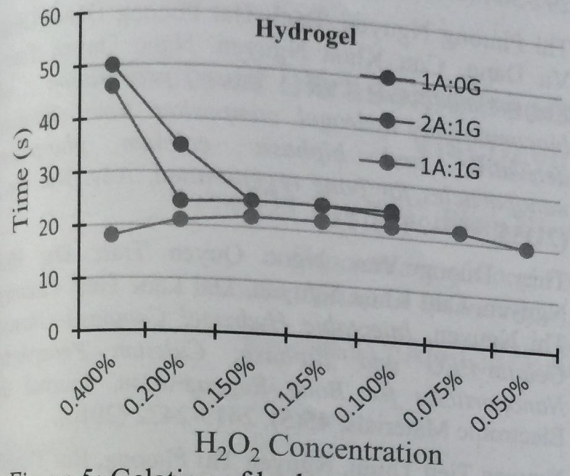

Figure 5: Gelation of hydrogel with 0.0125% HRP

Figure 6 shows the variation of gelation time when H_2O_2 concentration changes. The gelation time decreases when decreasing the concentration, and the gelation time is constant in the range of 0.1-0.15 % concentration. If H_2O_2 concentration is lower 0.1 %, gel formation does not occur. This performed in a similar manner to hydrogel.

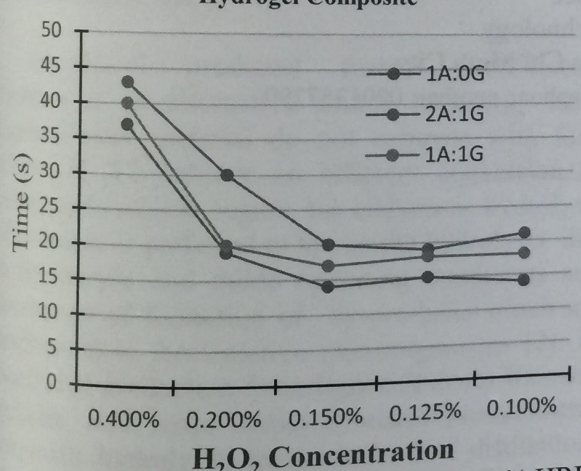

Figure 6: Gelation of NCgel with 0.0125 % HRP

3.4. Biodegradability

The enzyme collagenase is in the animal body: It is the main reagent of enzymatic hydrolysis, which breaks the peptide bonds of collagen. In this study, collagenase enzyme solution was used to survey the weight change of the hydrogel and NCgel through breaking the peptide bonds. Our previous study and other groups indicated that gelatin-based materials are rapidly degraded after 3 days when it is incubated in the collagenase solution [4]. This is the drawback of gelatin in bone regeneration. However, gelatin combined with alginate produced new materials has significantly long biodegradation time as seen in figures 7 and 8.

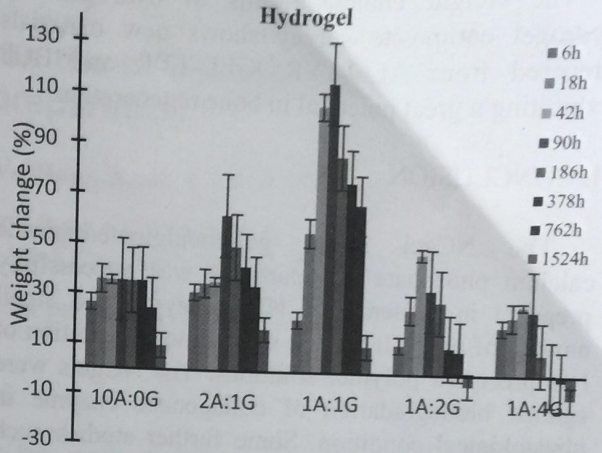

Figure 7: Weight change (%) of hydrogel ALG-TYR/GEL-TYR in collagenase solution

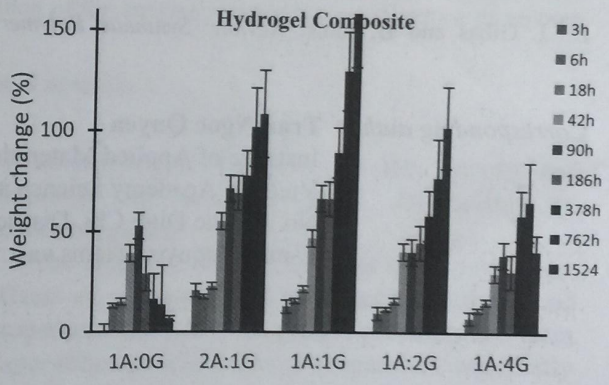

Figure 8: Weight change (%) of NCgel in collagenase solution

Weight of hydrogel and NCgel were changed in these experiments contributed by swelling ability of both hydrogel and NCgel. In hydrogel, the maximum water capacity occurs in the period from 42-90 h, hydrogel with 1A:1G ratio has highest capacity, get 50 times weight of the dried gel. Moreover, the figures also show that use of a suitable formulated GEL-TYR also enhanced swelling ratio of the materials. And some hydrogel samples begun degrading gradually after one month soaking. Compare with hydrogel, NCgel is more stable in collagenase solution and does not see the degradable weight after 1524 h (approximately two months). This is explained by the interaction between gelatin, alginate and BCP particle. Functional groups of gelatin (NH_2, OH, COOH), alginate (OH, COOH) and OH group of HAp in BCP have possibility to create hydrogen bond. In

addition, alginate and NH_2 group of gelatin interact with Ca^{2+} to stabilize structure.

The weight change results of hydrogel and hydrogel composite survey shows new materials prepared from ALG-TYR, GEL-TYR and BCP exhibiting a great potential in bone regeneration.

4. CONCLUSION

The NCgel based gelatin/alginate/biphasic calcium phosphate nanoparticles was successfully prepared in presence of HRP enzyme and small amount of H_2O_2. It takes a short time for gelation of two precursor polymer solutions. The NCgels were against biodegradation of collagenase enzyme in physiological condition. Some further studies such as cytocompatibility and biomineralization have conducted to confirm its potential application in bone regeneration.

REFERENCES

1. I. Gibas and H. Janik. *Review: Synthetic Polymer Hydrogels for Biomedical,* Chem. Technol., **(4)4**, 297-304 (2010).
2. Thi Phuong Nguyen, Bach Hai Phuong Doan, Dinh Vu Dang, Cuu Khoa Nguyen, Ngoc Quyen Tran. *Enzyme-mediated in situ preparation of biocompatible hydrogel composites from chitosan derivative and biphasic calcium phosphate nanoparticles for bone regeneration,* Adv. Nat. Sc., **(21)35**, 15012 (2011).
3. Thuy Duong Van, Ngoc Quyen Tran, Dai Hai Nguyen, Cuu Khoa Nguyen, Dai Lam Tran, Phuong Thi Nguyen. *Injectable Hydrogel Composite Based Gelatin-PEG and Biphasic Calcium Phosphate Nanoparticles for Bone Regeneration,* Journal of Electronic Materials, **45(5)**, 2415-2422 (2016).
4. Nguyen Tien Thinh, Nguyen Thi Phuong, Bui Thanh Thai, Nguyen Trong Tri, Nguyen Huynh Bach Son Long, Tran Quoc Son, Nguyen Tri Phu, Nguyen Cuu Khoa, Nguyen Dai Hai, Tran Ngoc Quyen. *Enzymatic preparation of modulated-biodegradable hydrogel nanocomposites based chitosan/gelatin and biphasic calcium phosphate nanoparticles*, Journal of Science and Technology, **55(1B)**, 185-192 (2017).

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