Viscoelasticity, mechanical properties, and *in vitro* biodegradation of injectable chitosan-poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/nanohydroxyapatite composite hydrogel

AHMED ABD EL-FATTAH^{1,2,*} and AYA MANSOUR¹

¹Department of Materials Science, Institute of Graduate Studies and Research, Alexandria University, Alexandria 21526, Egypt

²Department of Chemistry, College of Science, University of Bahrain, P.O. Box 32038, Sakhir, Kingdom of Bahrain

*Author for correspondence (a_fattaho@yahoo.com)

MS received 28 October 2017; accepted 26 January 2018; published online 3 December 2018

Abstract. A novel injectable composite hydrogel based on chitosan and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) reinforced with nanohydroxyapatite particles was synthesized. The chemical structure and morphology of the composite hydrogel were characterized. The composite hydrogel porosity, swelling, mechanical properties, viscoelasticity and *in vitro* biodegradation were also examined. Compared with the non-reinforced hydrogel, the composite hydrogel showed increased compressive strength, elastic modulus, viscous modulus, stiffness and had shear-thinning behaviour proving the injectability of the system. Swelling and biodegradation studies revealed that the composite scaffold possesses proper hydrophilicity and biodegradability. These properties make this composite hydrogel a promising injectable scaffold for bone regeneration.

Keywords. Injectable hydrogel; chitosan; poly(3-hydroxybutyrate-co-3-hydroxyvalerate); nanohydroxyapatite; viscoelasticity; bone regeneration.

1. Introduction

Bone is a naturally existing composite consists of a continuous phase of an organic matrix predominantly made of collagen type I, and a dispersed inorganic mineral phase of nanohydroxyapatite (nHA) crystals. This composite has remarkable mechanical, viscoelastic and biological properties [1,2]. However, bone may suffer certain defects as a result of disease, tumour resection and trauma [3,4]. As a consequence, tissue engineering has emerged to treat bone loss and repair. Several synthetic materials were investigated as potential substitutes for bone regenerative applications [5–7].

Injectable biodegradable hydrogels were extensively used in biomedical applications, such as tissue engineering and drug delivery because of their high tissue-like water content and mouldable features. Such injectable hydrogels are of particular significance because drugs, cells, proteins and bioactive agents can be essentially incorporated into polymer solutions before administration [8]. Moreover, their aqueous environment permits transportation of constituents, such as nutrients and cellular metabolites, which mimics biological tissue. Additionally, their administration into the body by simple injection avoids the need for invasive surgical interventions [9,10]. For use in bone tissue engineering, an ideal injectable hydrogel scaffold should be of low viscosity prior to injection, rapidly gels in the human body, biocompatible

and bioactive to enhance tissue formation. Moreover and most important is it should possess appropriate mechanical and viscoelastic properties comparable to the native bone [11,12].

An attractive candidate hydrogel for biomedical applications is chitosan (CS), a natural polymer, obtained from the deacetylation of chitin. It has unique properties, including low cost, biodegradability, biocompatibility, non-antigenicity, antibacterial effect and flexibility in hydrated environments [13–15]. However, the major limitations of CS hydrogels are the poor mechanical strength and bioactivity to promote formation of new bone upon implantation [16].

It was reported in previous studies that the incorporation of bioceramics can significantly improve mineralization and cell activities on polymeric hydrogels, showing osteoinductivity and/or osteoconductivity for bone regeneration applications [17,18]. Among the available bioceramics, nHA particles have received great attention in bone regeneration due to its excellent bioactivity, biocompatibility and potential osteogenic effect [19,20].

Therefore, it is desirable to develop a composite hydrogel scaffold with the favourable properties of both CS and nHA. The designed composites are likely to display enhanced bioactivity and sufficient mechanical strength, which will be of great importance for tissue engineering application. It was reported that CS/HA composites show good biocompatibility and osteoconductivity [21–23]. On the other hand, a number

of other polymers such as polylactic acid (PLA) and alginate were used in the CS/HA system to enhance the compressive strength significantly [24,25].

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a natural origin, biodegradable, biocompatible polyester with piezoelectric behaviour and suitable mechanical properties, which is widely used in biomedical applications [26]. Moreover, PHBV does not produce harmful acidic products like PLA during the biodegradation process, which may be harmful for human tissues [27]. To increase the mechanical strength of CS/HA composite hydrogel scaffolds, we introduced PHBV into the CS/HA system. To our knowledge, no previous work has investigated CS-PHBV hydrogel incorporated with nHA particles to promote compressive strength and bioactive properties of the hydrogel-based composite.

In this study, injectable CS-PHBV/nHA composite hydrogel was synthesized by the regeneration chemistry approach without any chemical crosslinkers. The chemical interaction between the inorganic phase and the organic matrix as well as the mechanical and viscoelastic properties of the resulting composite hydrogel were investigated. This work would provide insight into the designing of a promising injectable hydrogel scaffold through simple technique, which would find potential application in bone tissue engineering.

2. Materials and methods

2.1 Materials

PHBV in a fine powder form was provided by Tainan Biologic Materials Co., China, with molecular weight of 400,000 Daltons and contains 3% mole of β -hydroxyvalerate. CS, 93% deacetylation and calcium chloride anhydrous were obtained from Oxford Co., India. Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) and ammonium dihydrogen phosphate ($(\text{NH}_4)_2\text{H}_2\text{PO}_4$) were purchased from Sigma Aldrich Chemical Co., Germany, and were used as the starting materials for Ca and P precursors, respectively. Ammonia, methanol, glacial acetic acid, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, sodium chloride and potassium chloride and phosphate-buffered saline (PBS, pH 7.4) as well as lysozyme (10^4 units ml^{-1}) were purchased from Sigma Aldrich Chemical Co., Germany.

2.2 Synthesis of nHA particles

The nHA particles were synthesized by the wet chemistry method as described in the previous work [11]. In the synthesis process, solution A of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.1 M) and solution B of $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ (0.06 M) were prepared. Solution B was added dropwise at a constant rate (3 ml min^{-1}) to solution A under vigorous stirring at 37°C , whereas aqueous ammonia was used to adjust the pH of the solution at 11. The obtained precipitated powder was aged overnight, filtered, washed with deionized water and finally dried at 200°C for 24 h.

2.3 Preparation of composite hydrogel

CS-PHBV control hydrogel was synthesized by the regeneration chemistry method according to Nair *et al* [28]. CS solution was prepared by dissolving CS (0.5 g) in acetic acid (100 ml, 1%) under constant stirring. PHBV solution was prepared by dissolving PHBV (0.5 g) in saturated CaCl_2 -methanol solvent (100 ml) at 60°C . The CS solution (0.5% w/v) was mixed with PHBV solution (0.5% w/v) with a volume ratio of (75:25), respectively, and neutralized by the addition of NaOH solution (1 N). The mixed solutions were stirred for 2 h till a gel is formed, centrifuged at 8000 rpm for 10 min, and washed with distilled water to remove the traces of CaCl_2 and methanol. Prior to characterization, the obtained hydrogel was cooled to the temperature of -25°C overnight. Then, it was lyophilized under liquid nitrogen in a freeze drier for 24 h. For the synthesis of CS-PHBV/nHA composite hydrogel, the nHA particles (0.05 g) were added to the CS solution and the previous steps were repeated.

2.4 Characterization of nHA particles and composite hydrogel

2.4a FTIR: Fourier transform infrared (FTIR) spectroscopy is an appropriate tool to examine polymer-particle interaction. FTIR spectra for the prepared samples were recorded using Perkin-Elmer FTIR spectrometer throughout the range from 4000 to 350 cm^{-1} for spectroscopic identification of the functional groups present in the nHA particles, CS-PHBV hydrogel and CS-PHBV/nHA composite hydrogel. The samples were prepared using the KBr disc technique in which the powder samples (5 mg) were compressed with KBr (200 mg) under hydraulic pressure to form a disk with a diameter of 13 mm.

2.4b EDS: Semi-quantitative analysis of the chemical composition of the prepared nHA particles was conducted by energy dispersive X-ray (EDS). Analysis was performed on uncoated powder samples at 15 kV for 60 s. The Ca/P molar ratio of the sample was determined as the average of five runs.

2.4c XRD: Structural analyses of the nHA particles were performed by powder X-ray diffraction (XRD) measurements using a Diffractometer (XRD-7000, Shimadzu, Japan). The X-ray beam was $\text{CuK}\alpha$ radiation ($\lambda = 0.1540 \text{ nm}$), operated at 40 kV and 38 mA. The XRD pattern was recorded in the 2θ range from 20 to 60° with a scanning rate of 5° per min. The crystalline phase of the nHA particles was identified and compared to the literature reports as well as the Joint Committee on Powder Diffraction Standards (JCPDS) for HA with the PDF number 74-0566.

2.4d SEM: The morphology of all the samples were examined using scanning electron microscopy (SEM) (JEOL 5300-JSM, Japan) operated between 15 and 30 keV. The

samples were frozen under liquid nitrogen, mounted and sputter-coated with gold to a thickness of 400 Å in a sputter-coating unit (JFC 1100 E) prior to imaging by SEM.

2.4e TEM: The size and morphology of nHA particles were studied using a JEOL-JEM-100CX ((Japan) transmission electron microscope (TEM)). Few drops of the nHA particles (dispersed in ethanol) were mounted on carbon-coated copper grids, double stained with freshly prepared uranyl acetate for 20 min, then, with lead citrate for 2–5 min prior to imaging by TEM.

2.4f Porosity: The porosity of the CS-PHBV hydrogel and CS-PHBV/nHA composite hydrogel were determined at 37°C by the liquid displacement method [29]. Cylindrical hydrogel scaffolds of different weights between 1.18 and 0.5 g were immersed individually in a graduated cylinder containing distilled water (25 ml) for 24 h to achieve the equilibrium. Then, the hydrogel scaffolds were freeze-dried and the final weight recorded. The porosity of the scaffolds was calculated according to equation (1) [26,29]:

$$\text{Porosity (\%)} = \frac{W_2 - W_1}{\rho V_s} \times 100, \quad (1)$$

where W_1 and W_2 represent the weight of the hydrogel scaffolds before and after immersion in the distilled water, ρ the density of distilled water and V_s the volume of the dried hydrogel scaffold. Porosity value was expressed as mean $\pm$ SD ($n = 5$).

2.4g Swelling ratio: CS-PHBV hydrogel and CS-PHBV/nHA composite hydrogel samples of different weights range between 1.18 and 0.5 g were incubated in PBS (pH = 7.4) at 37°C for two weeks to determine their water uptake. Samples were removed at the predetermined time periods and the excess water on the surface was wiped out using a tissue paper and then weighed. The swelling ratio of the hydrogels was determined using equation (2) [30,31].

$$\text{DS (\%)} = \frac{W_w - W_d}{W_d} \times 100, \quad (2)$$

where DS is the degree of swelling, while W_d and W_w are the dry and wet weights of the samples, respectively. Swelling ratios are expressed as a mean $\pm$ SD ($n = 5$).

2.4h Mechanical properties: The compressive stress and strain of the hydrogel samples were measured using a mechanical testing machine (DMA 7e, Perkin Elmer). In this test, the hydrogel samples were shaped as cylinders without lyophilization. The samples used were (1.5 cm diameter $\times$ 1 cm height) in dimension. Each hydrogel was tested until it fractured. The samples were investigated and the data measured as a mean $\pm$ SD ($n = 5$).

2.4i Rheological measurements: Rheological measurements of the hydrogel samples were carried out using a strain-controlled Rheometer (Anton Paar Rheoplus V3.62 – MCR 302) with a temperature control. A dynamic frequency sweep test was performed with a strain amplitude of $\gamma_0 = (1\%)$ at an angular frequency range of 1–100 rad s^{-1} at 37°C. The complex viscosity (η^*), phase shift angle (δ), loss tangent ($\tan \delta$), elastic modulus (G') as well as the viscous modulus (G'') were recorded as a function of angular frequency (ω). The magnitude of the complex shear modulus (G^*) was calculated using equation (3) [28]:

$$|G^*| = |\eta^*| \times \omega. \quad (3)$$

2.4j In vitro enzymatic biodegradation: The *in vitro* biodegradation of the hydrogel scaffolds was studied by immersion in PBS (pH = 7.4) with lysozyme at $37.0 \pm 0.5^\circ\text{C}$ [32]. Pre-weighed (W_i) dry scaffolds were immersed in the PBS and incubated into a shaker with 50 rpm for 14 days. Then, the scaffolds were taken out from the medium at 1, 7 and 14 days and were washed with deionized water to remove the adsorbed ions and salts on surface and then, the samples were freeze-dried. PBS was replaced every 3 days. Dry weight of the scaffolds was recorded as W_d . The biodegradation percentage was calculated using equation (4) [32]:

$$\text{Biodegradation (\%)} = \frac{W_i - W_d}{W_i} \times 100. \quad (4)$$

2.4k Statistical analysis: The *t*-test was carried out using the statistical package for the social sciences (SPSS) for comparisons between the CS-PHBV hydrogel and CS-PHBV/nHA composite hydrogel. The level of significance was set at the value of $P < 0.05$.

3. Results and discussion

3.1 Characterization of nHA particles

The characterization results of the prepared nHA particles are displayed in figure 1. As shown in figure 1a, the SEM image of the prepared nHA particles exhibited nearly a regular rod-like structure with uniform particle size. The diameters of these rods were about 13 nm and the lengths were nearly 80 nm as revealed by TEM image (figure 1b). The rod-like structure of nHA particles is proved to be greatly advantageous to enhance the mechanical properties of biomedical implants [22]. EDS analysis (figure 1c) revealed a Ca/P ratio of 1.7 ± 0.10 , which is in good agreement with the actual value of HA [16]. Figure 1d shows the XRD spectrum of the nHA, which indicates the main characteristic diffraction peaks of HA crystals. The observed XRD spectrum was compared to the HA reference data (JCPDS-PDF no. 74-0566), where both spectra matched closely and exhibited a good coincidence without additional phases [7].

Figure 1. Characterization of nHA: (a) SEM image, (b) TEM image, (c) EDS chart and (d) XRD spectrum.

3.2 Hydrogel preparation

CS-PHBV control hydrogel and CS-PHBV/nHA composite hydrogel were successfully prepared by simple regeneration chemistry. The CS and PHBV solutions were mixed together and physical crosslinking of the polymer chains were occurred. The rod-like nHA particles were entrapped in the hydrogel matrix, thereby nHA particles were physically incorporated into the polymer chains. In this work, the amount of CS, PHBV and nHA particles were fixed at 75, 25 and 5%, respectively. These values were selected because they represent an optimum performance of mechanical and viscoelastic properties of the scaffold as well as ease of injectability [28]. Although higher concentrations of nHA particles have provided better bioactivity, nevertheless, concentrations exceeding the selected value were denounced to avoid aggregation of nHA particles, which in turn may deteriorate the overall mechanical properties of the composite and hinder hydrogel injectability [14,33].

3.3 Interaction between polymer matrix and inorganic phase

FTIR data revealed important information about the interactions between the CS-PHBV polymer matrix and the nHA inorganic phase. Figure 2 shows the FTIR spectra of the nHA, CS and PHBV as well as their control and composite

Figure 2. FTIR spectra of nHA, CS and PHBV as well as their CS-PHBV hydrogel and CS-PHBV composite hydrogel.

hydrogels. Then, nHA spectrum showed absorption band at 3452 cm⁻¹, which is attributed to the hydroxyl (OH) group stretching vibration [34]. The bands at 1637 and

Figure 3. SEM images of (a) CS-PHBV, (b) CS-PHBV/nHA and (c) CS-PHBV/nHA after sonication in water for 10 min prior to characterization.

1386 cm^{-1} represented the bending vibration modes of the water crystals retained by hydrogen bonds on the nHA surface. The peaks appeared at 1035 and 960 cm^{-1} were corresponding to the stretching vibrations of the phosphate group (P-O) [35]. Moreover, the absence of the nitrate (NO_3^-) group bands at 820 and 1380 cm^{-1} indicated that no residual NO_3^- was associated with the nHA particles [19]. The appearance of the carbonate (CO_3^{2-}) group stretching and bending vibration bands at 1421 and 872 cm^{-1} , suggests the occurrence of carbonate substitution [36], which enhances the bioactivity of the nHA particles as the composition of the biological apatite includes CO_3^{2-} at about 4.5% [37]. These results indicated the successful preparation of pure nHA particles.

It was generally known that CS showed combined absorption peaks at $3200\text{--}3500\text{ cm}^{-1}$ corresponding to stretching vibrations of the amino ($-\text{NH}_2$) and OH groups [16,38]. The band at around 1651 cm^{-1} can be assigned to the stretching vibration of amide II ($-\text{NH}$) corresponding to the ($\text{C}=\text{O}$) stretching mode. The spectrum of PHBV revealed a characteristic band at 1728 cm^{-1} due to the ($\text{C}=\text{O}$) stretching vibration mode. All the characteristic peaks of CS and PHBV were observed in the FTIR spectrum of CS-PHBV hydrogel. The interaction between CS and PHBV probably occurs through hydrogen bonding among ($\text{C}=\text{O}$) groups of PHBV with (OH) and (NH) groups present in CS. This result indicated that there was no chemical bonding between PHBV and CS in the formed hydrogel and

Figure 4. Swelling ratio of CS-PHBV and CS-PHBV/nHA composite hydrogels in distilled water at 37°C .

they are only physically coexist [31]. The FTIR spectrum of CS-PHBV/nHA composite hydrogel exhibited no significant differences compared to the FTIR spectrum of CS-PHBV, as shown in figure 2. However, the increased intensity of the characteristic peak of the CS at 1651 cm^{-1} indicated the strong interaction through formation of hydrogen bonding between (OH) groups in nHA particles and the ($\text{C}=\text{O}$) groups in hydrogel molecular chains [39].

Figure 5. (a) Mechanical properties for the CS–PHBV and CS–PHBV/nHA composite hydrogels: with $P = 0.022$ as well as the compressed hydrogels photographs of the mechanical test: (b) before testing, (c) top-view after testing and (d) side-view after testing.

3.4 Hydrogel morphology

Figure 3 shows SEM images of the control and composite hydrogels. As shown in figure 3a and b, the SEM images revealed that the hydrogels with and without nHA particles had the same internal structure. Besides, the hydrogels are porous with small pores that were spread over the entire structure. Moreover, figure 3c revealed a uniform and homogeneous distribution of nHA particles throughout the composite hydrogel. No delamination was observed between the inorganic particles and the organic matrix indicating a close bonding between them, which possibly will help to improve the mechanical properties [25].

3.5 Porosity and swelling ratio

The calculated porosity values from equation (1) of the control and composite hydrogels were about 35.9 ± 1.28 and $16.04 \pm 1.63\%$, respectively. It was noticed that the incorporation of nHA particles in the composite hydrogel triggered a reduction in porosity by 55.3%, which is in accordance with the previously reported data for other hydrogels with different bioceramics [40].

Figure 4 shows a swelling behaviour of the control and composite hydrogel scaffolds due to water uptake in distilled water at 37°C over two weeks. In general, the swelling ratio of the composite scaffold was decreased due to the addition of nHA particles. More specifically, after 14 days, the swelling has induced a mass increase of $125 \pm 5\%$ in the control hydrogel, while the swelling of the composite hydrogel was restricted to $110 \pm 5\%$ i.e., 12% decrease in the swelling ratio. This phenomenon may be related to the physical trapping of nHA particles within the CS–PHBV hydrogel, which

Figure 6. Elastic (G'), viscous (G'') and complex shear (G^*) moduli of the CS–PHBV and CS–PHBV/nHA composite hydrogels as a function of the angular frequency.

is due to the formation of hydrogen bonds between the nHA surfaces and the polymer matrix. It is worth mentioning that an increase in the swelling ratio negatively affects the mechanical properties of hydrogel scaffolds and also influences the cell migration and neovascularization [41]. Consequently, this reduction in swelling ratio was expected to induce a greater strength of the CS–PHBV composite hydrogel. Moreover, the composite scaffold is hydrophilic, thus, it can be potentially applied for tissue engineering applications as the hydrophilicity of the scaffold will enable the absorption of body fluid,

Figure 7. (a) Phase shift angle and (b) loss tangent of the CS-PHBV hydrogel and CS-PHBV/nHA composite hydrogel as a function of the shear rate with $P = 0.030$.

which consists of mainly water and is important for the diffusion of nutrient and metabolites [24].

3.6 Mechanical properties

The mechanical properties of the control and composite hydrogels were characterized and displayed in figure 5. It was found that the incorporation of nHA particles in the composite hydrogel increased the ultimate compressive strain, ultimate compressive strength and Young's modulus by 16.4, 88.4 and 79.5%, respectively, compared to the control hydrogel. The improvement in mechanical properties of CS-PHBV/nHA composite hydrogel could be attributed to: (i) the strong interfacial interaction between inorganic and organic phases gained from the formation of hydrogen bond; (ii) the introduction of PHBV, which has a good mechanical property as an organic reinforcement phase; (iii) the incorporation of rod-like nHA particles as an inorganic reinforcement phase; and (iv) the correct inorganic/organic ratio [25]. Furthermore, all the hydrogel scaffolds retain a flat sheet shape and did not spatter randomly by the end of the compression testing as shown in figure 5b, c and d. Compared to other reported organic/inorganic composite hydrogels, the CS-PHBV/nHA composite hydrogel has markedly superior mechanical properties for orthopaedic applications [42].

3.7 Viscoelastic behaviour studies

Ideally, an injectable hydrogel should perform as a low-viscosity liquid through application, but as an elastic solid when filling the defect with a fast conversion between both the states [43]. Viscoelasticity of the injectable hydrogels was evaluated using dynamic frequency test to measure the elastic (G'), viscous (G'') and complex shear (G^*) moduli, which are shown in figure 6. First, it is important to note that G' is much larger than G'' over all the frequency ranges studied for both hydrogels, which confirm that the hydrogels are highly

elastic. Second, it can be seen that G' and G'' increased with the increase in the angular frequency for both tested samples. This may be attributed to the intertwining of the polymeric chains that resulted in restriction of the chains motion at high frequency, consequently, inability of the chains to relax and return to their original position [41]. Third, G' , G'' and G^* of the composite hydrogel were higher than that of the control hydrogel at all the frequencies. This may be explained by the incorporation of the rod-like nHA particles occupying the empty voids within the polymeric scaffold, which was strongly correlated with greater stiffness and rigidity and reduced elasticity and viscosity [44]. Finally, it is worth mentioning that the G^* values of the composite hydrogel were 17.1 ± 5.6 kPa at 1 rad s^{-1} , 18.9 ± 7.2 kPa at 10 rad s^{-1} and 21.8 ± 9.4 kPa at 100 rad s^{-1} , which were in agreement with the previously reported data of G^* values for human intervertebral disc's lumbar nucleus pulposus [45].

Furthermore, the phase shift angle and loss tangent (figure 7) for the control and composite hydrogels were around 8° and less than unity, respectively. These results indicated that the hydrogels were of solid, dominating with a fluid component [46].

The change in complex viscosity with respect to the shear rate gives the flow curves of the control and composite hydrogels is displayed in figure 8a. The flow curves of both the hydrogels showed that the complex viscosity decreased as the shear rate increased, which indicates that the hydrogels are non-Newtonian fluids and revealed a shear-thinning behaviour [47]. This is the most critical property for an injectable material because as the shear is applied to the hydrogels by the syringe plunger, it will smoothly flow out of the syringe [48]. In addition, the yield stress is a good indicator of the hydrogel injectability, as the hydrogel will not flow through the syringe nozzle until sufficient yield stress is applied. The values of yield stress for the control and composite hydrogels were 7.4 and 16.5 kPa, respectively (figure 8b). As represented in figure 8c and d, the control

Figure 8. (a) Complex viscosity and (b) shear stress of the CS-PHBV and CS-PHBV/nHA composite hydrogels as a function of the shear rate with $P = 0.030$ as well as (c, d) photographs representing the injectability of the hydrogels.

and composite hydrogels showed a smooth injectability without any discontinuity in the flow, through the nozzle of the syringe. Moreover, the hydrogels reserved their shape and did not flow on their own after being emerged from the syringes. This showed that the hydrogels could be simply injected into inaccessible sites and it could adapt to the defect shape by applying the required shear. Furthermore, after placing into the defect, the composite hydrogel has enough adhesive nature to the bone defect margins and thus, will stay

without flowing into the neighbouring tissue at normal body temperature.

3.8 *In vitro* biodegradation behaviour

The biodegradation of the control and composite scaffolds was studied in PBS-lysozyme solution for 14 days. In our study, the presence of nHA in the scaffold exhibited insignificant change in degradation rate (figure 9). It can be noticed

Figure 9. *In vitro* biodegradation of the CS-PHBV and CS-PHBV/nHA composite hydrogels in PBS-lysozyme environment with $P = 0.030$ (mean $\pm$ SD), $n = 5$.

that both the scaffolds showed a lower biodegradation rate on the first day followed by a higher biodegradation rate on the 7th and 14th days (figure 9). More specifically, the control scaffold showed a biodegradation rate of 7.5, 56.8 and 96.2% on 1st, 7th and 14th day, respectively. While the composite scaffold resulted in a lagging biodegradation rate of 4.3, 38.7 and 85.3% on 1st, 7th and 14th day, respectively. This indicates that the addition of nHA particles reduced the rate of biodegradation of the CS-PHBV/nHA composite scaffold to a great extent. This result might be attributed to the formation of more stable and compact scaffold structure formed by the interactions among the polymers (CS, PHBV) and nHA particles. The biodegradation result is consistent with other studies in which the reduced biodegradation was observed in nHA-based scaffolds [29,49]. The slower biodegradation rate of the scaffold might be valuable as it allows the scaffold to maintain its mechanical integrity and provide sufficient time for bone growth throughout the implant [49].

4. Conclusions

A new injectable CS-PHBV/nHA composite hydrogel was successfully synthesized without any chemical crosslinkers using regeneration chemistry. The control CS-PHBV and composite CS-PHBV/nHA hydrogels were characterized using SEM and FTIR, which confirmed the incorporation and uniform dispersion of rod-like nHA particles into CS-PHBV polymer matrix. The swelling ratio, porosity, mechanical properties and viscoelasticity as well as *in vitro* biodegradation of the hydrogels were investigated. Upon addition of rod-like nHA particles in the composite hydrogel formulations, the experimental results indicated that the porosity was reduced by 55.3% and the swelling ratio was decreased by 12% after immersion in distilled water for one day, whereas the compressive strength and compressive strain

were enhanced by 88.4 and 16.4%, respectively. Additionally, the G' was much larger than G'' at all the frequency range studied, which exhibits a self-standing elastic composite hydrogel. The composite hydrogel exhibits a shear-thinning behaviour and flows under shear stress. Finally, the biodegradation rate after immersion in physiological environment for two weeks was decreased by 27.3%. Thus, the optimized CS-PHBV/nHA composite hydrogel demonstrated good scaffold properties suggesting promising applications in bone-tissue engineering and regeneration. Experiments on the *in vivo* biocompatibility and osteogenic differentiation capacity of the scaffold will be conducted in the near future.

Acknowledgements

We acknowledge Sherif Kandil and Labiba El-Khordagui at Alexandria University, Egypt as well as El-Refaie Kenawy at Tanta University, Egypt, for their scientific guidance and moral support throughout the work.

References

- [1] Kumar A, Negi Y S, Choudhary V, Bhardwaj N K and Han S S 2017 *Int. J. Polym. Anal. Charact.* **22** 139
- [2] Chamundeeswari M, Kumar B S, Muthukumar T, Muthuraman L, Sai K P and Sastry T P 2013 *Bull. Mater. Sci.* **36** 1165
- [3] Li X, Wang L, Fan Y, Feng Q, Cui F Z and Watari F 2013 *J. Biomed. Mater. Res. A* **101** 2424
- [4] Ding Z, Han H, Fan Z, Lu H, Sang Y, Yao Y *et al* 2017 *ACS Appl. Mater. Interfaces* **9** 16913
- [5] Salgado A J, Coutinho O P and Reis R L 2004 *Macromol. Biosci.* **4** 743
- [6] Seyednejad H, Gawlitta D, Dhert W J, Van Nostrum C F, Vermonden T and Hennink W E 2011 *Acta Biomater.* **7** 1999
- [7] Hassan M N, Mahmoud M M, El-Fattah A A and Kandil S 2016 *Ceram. Int.* **42** 3725
- [8] Sivashanmugam A, Kumar R A, Priya M V, Nair S V and Jayakumar R 2015 *Eur. Polym. J.* **72** 543
- [9] Neffe A T, Loebus A, Zaupa A, Stoetzel C, Müller F A and Lendlein A 2011 *Acta Biomater.* **7** 1693
- [10] Seeherman H and Wozney J M 2005 *Cytokine Growth Factor Rev.* **16** 329
- [11] Slaughter B V, Khurshid S S, Fisher O Z, Khademhosseini A and Peppas N A 2009 *Adv. Mater.* **21** 3307
- [12] Lo C M, Wang H B, Dembo M and Wang Y L 2000 *Biophys. J.* **79** 144
- [13] Zhou H Y, Jiang L J, Cao P P, Li J B and Chen X G 2015 *Carbohydr. Polym.* **117** 524
- [14] Nawrotek K, Tylman M, Rudnicka K, Balcerzak J and Kamiński K 2016 *Carbohydr. Polym.* **136** 764
- [15] Karbasi S and Alizadeh Z M 2017 *Bull. Mater. Sci.* **40** 1247
- [16] Wang Q and Chen D 2016 *Carbohydr. Polym.* **136** 1228
- [17] Liu B, Yao Y, Su J and Xiao J 2015 *Mater. Today Proc.* **2** 295
- [18] Kumar B S, Hemalatha T, Deepachitra R, Raghavan R N, Prabu P and Sastry T P 2015 *Bull. Mater. Sci.* **38** 259
- [19] Bakan F, Laçın O and Sarac H 2013 *Powder Technol.* **233** 295

- [20] Fu S, Guo G, Gong C, Zeng S, Liang H, Luo F *et al* 2009 *J. Phys. Chem. B* **113** 16518
- [21] Pangon A, Saesoo S, Saengkrit N, Ruktanonchai U and Intasanta V 2016 *Carbohydr. Polym.* **144** 419
- [22] Dhivya S, Saravanan S, Sastry T P and Selvamurugan N 2015 *J. Nanobiotechnol.* **13** 1
- [23] Liu X, Chen Y, Huang Q, He W, Feng Q and Yu B 2014 *Carbohydr. Polym.* **110** 62
- [24] Sharma C, Dinda A K, Potdar P D, Chou C F and Mishra N C 2016 *Mater. Sci. Eng. C Mater. Biol. Appl.* **64** 416
- [25] Cai X, Tong H, Shen X, Chen W, Yan J and Hu J 2009 *Acta Biomater.* **5** 2693
- [26] Sankar D, Chennazhi K P, Nair S V and Jayakumar R 2012 *Carbohydr. Polym.* **90** 725
- [27] Shishatskaya E I, Volova T G, Puzyr A P, Mogilnaya O A and Efremov S N 2004 *J. Mater. Sci. Mater. Med.* **15** 719
- [28] Nair M B, Baranwal G, Vijayan P, Keyan K S and Jayakumar R 2015 *Colloids Surf. B Biointerfaces* **136** 84
- [29] Peter M, Binulal N S, Soumya S, Nair S V, Furuike T, Tamura H *et al* 2010 *Carbohydr. Polym.* **79** 284
- [30] Devine D M and Higginbotham C L 2005 *Eur. Polym. J.* **41** 1272
- [31] Zhong X, Ji C, Chan A K, Kazarian S G, Ruys A and Dehghani F 2011 *J. Mater. Sci. Mater. Med.* **22** 279
- [32] Kumar P S, Srinivasan S, Lakshmanan V K, Tamura H, Nair S V and Jayakumar R 2011 *Carbohydr. Polym.* **85** 584
- [33] Thorpe A, Creasey S, Sammon C and Le Maitre C 2016 *Eur. Cells Mater.* **32** 1
- [34] Hassan M N, Mahmoud M M, El-Fattah A A and Kandil S 2015 *J. Sol-Gel Sci. Technol.* **76** 74
- [35] Varma H K and Babu S S 2005 *Ceram. Int.* **31** 109
- [36] Ślósarczyk A, Paszkiewicz Z and Paluszkiwicz C 2005 *J. Mol. Struct.* **744** 657
- [37] Murugan R and Ramakrishna S 2005 *Compos. Sci. Technol.* **65** 2385
- [38] Murthy B S, Ramanathan G and Sivagnanam U T 2017 *Bull. Mater. Sci.* **40** 645
- [39] Sukhodub L B, Yanovska G O, Kuznetsov V M, Martynyuk O O and Sukhodub L F 2016 *J. Nano-Electron. Phys.* **8** 1032
- [40] Roberts J J, Earnshaw A, Ferguson V L and Bryant S J 2011 *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **99** 158
- [41] Killion J A, Kehoe S, Geever L M, Devine D M, Sheehan E, Boyd D *et al* 2013 *Mater. Sci. Eng. C Mater. Biol. Appl.* **33** 4203
- [42] Sarker A, Amirian J, Min Y K and Lee B T 2015 *Int. J. Biol. Macromol.* **81** 898
- [43] Gantar A, Drnovšek N, Casuso P, Pérez-San Vicente A, Rodriguez J, Dupin D *et al* 2016 *RSC Adv.* **6** 69166
- [44] Gaharwar A K, Rivera C P, Wu C J and Schmidt G 2011 *Acta Biomater.* **7** 4139
- [45] Iatridis J C, Setton L A, Weidenbaum M and Mow V C 1997 *J. Biomech.* **30** 1005
- [46] Bader R A 2008 *Acta Biomater.* **4** 967
- [47] Arun Kumar R, Sivashanmugam A, Deepthi S, Iseki S, Chennazhi K P, Nair S V *et al* 2015 *ACS Appl. Mater. Interfaces* **7** 9399
- [48] Kumar P S, Srinivasan S, Lakshmanan V K, Tamura H, Nair S V and Jayakumar R 2011 *Int. J. Biol. Macromol.* **49** 20
- [49] Peter M, Binulal N S, Nair S V, Selvamurugan N, Tamura H and Jayakumar R 2010 *Chem. Eng. J.* **158** 353