Published in final edited form as:

Mater Sci Eng C Mater Biol Appl. 2018 March 01; 84: 281–289. doi:10.1016/j.msec.2017.11.001.

Extended release of proteins following encapsulation in hydroxyapatite/chitosan composite scaffolds for bone tissue engineering applications

Declan M. Devine^{1,2}, Eilish Hctor¹, Jessica S. Hayes³, Eoin Sheehan⁴, and Christopher H. Evans²

¹Materials Research Institute, Athlone Institute of Technology, Athlone, Ireland ²Rehabilitation Medicine Research Center, Mayo Clinic, Rochester, MN, USA ³College of Medicine, Nursing and Health Science, National University of Ireland, Galway, Ireland ⁴Orthopaedic Department, Midlands Regional Hospital, Tullamore, Ireland

Introduction

The long bones of the body have considerable regenerative capacity and bone is one of the few organs that can heal spontaneously without the formation of scar tissue[1]. This innate ability to regenerate stems from the constant remodelling that bones undergo as a result of everyday loading [2]. However, clinical problems arise when the demands of the injury exceed the healing capacity of the bone. A fracture normally heals in 6–8 weeks[3]. In 5–10% of fractures, however, healing is delayed or absent; the incidence increases when an open fracture occurs. If the bone does not heal it is considered a non-union which, in severe cases, can lead to amputation[4]. Another clinical problem occurs with critical sized osseous defects which result from the loss of a large segment of bone which exceeds its natural healing capacity[5]. These circumstances can result from a variety of incidents, such as high speed trauma, debridement of tissue following infection or for iatrogenic reasons, such as tumor resection.

Bone grafting is the treatment of choice for these conditions and an estimated 2.2 million such procedures occur worldwide each year[6]. The gold standard graft uses bone taken from the patient's own non-essential bone stock, typically the iliac crest. However, despite its success and widespread use, autografting has several inherent disadvantages. These include the need for a second surgery to harvest the bone, which has a high incidence of morbidity and increases the risk of infection, and limited bone stock, especially in patients suffering from osteoporosis or patients that have already undergone a similar procedure[7]. Additionally, it has been reported that, depending on the location, up to 25% of bone grafts

Correspondence: Dr. D. Devine, Materials Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Ireland N37 HD68, Tel: 00353 90 6468291.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

resorb within 5 years [8]. To circumvent these issues allogeneic grafts are sometimes used. However, they are less osteogenic than autologous bone grafts and they carry an increased risk of infection and disease transmission. Thus there is a clear clinical need to develop superior alternatives to bone grafting[1]. Tissue engineering offers one approach to addressing this need.

Constructs for bone tissue engineering usually include bone graft substitute materials that combine mechanical strength with osteoinductive or osteoconductive properties. Faour *et al.* [9]define the ideal bone graft substitute as a material that is biocompatible, bioresorbable, osteoconductive, osteoinductive, structurally similar to bone, easy to use and cost effective. Tissue engineering traditionally involves harvesting the patient's cells, expanding them as monolayers and, when sufficient numbers of cells have been obtained, seeding them onto a 3D matrix which is subsequently cultured in a bioreactor with signalling molecules prior to re-implantation. However, if the need to harvest, culture and reimplant autologous cells were eliminated, the patient could be treated immediately in a single surgery[10].

A promising acellular strategy centers on the development of scaffolds impregnated with osteogenic factors which are released for an extended period of time after implantation, thereby promoting bone formation by endogenous osteoprogenitor cells. While this approach appears simple, the difficulty in achieving this can be illustrated by reference to current growth factor delivery systems used clinically for bone healing. These are available as Medtronic's Infuse® (Bone morphogenetic protein (BMP)-2) and Stryker's OP-1® (BMP-7). Both products come with a two part system where the growth factor is reconstituted and mixed with the carrier collagen sponge (Infuse®) or powder (OP-1®). This results in the growth factor being only loosely bound to the scaffold from which it washes away intraoperatively. Once removed from the scaffold the plasma half-life of BMP-2 is approximately 10 min[11], To compensate for their short dwell-time, massively supraphysiological doses of BMPs are used. Not only are these marginally effective in clinical practice, but they produce several adverse side effects, some of them severe[12,13]. Another failing of the collagen scaffold is its inability to contribute to load bearing, which itself can lead to non-unions[14]. Hence, it clear that novel approaches are needed to improve the scaffolds used for bone tissue engineering. Here we describe a new strategy for extending the period of protein release within osseous lesions, by the covalent attachment of the proteins to scaffolds.

In this context, we developed a novel composite scaffold as a delivery vehicle. The composite scaffold was designed to be biomimetic, consisting of hydroxyapatite (HAp) which is similar to the mineral component of bone, in combination with the natural polymer chitosan (CS) [15]. HAp was used to enhance the load bearing capacity of the composite utilizing nanocomposite technology to produce a material which mimics the fine structure of natural bone[16]. CS was used as an analog of collagen, which is the polymer phase of bone, and as a molecule to which proteins could be covalently bound[11]. CS is a deacylated form of chitin, an abundant naturally occurring polymer derived from the exoskeleton of crustaceans and insects; it is a waste product of the food industry. Additionally, it has reported antimicrobial properties and is osteoconductive [17]. The use of HAp to enhance the properties of CS has previously been reported. However, these composites are frequently

produced using methods such as the in situ chemical method or freeze drying[16,18]. The novelty of the current approach is the chemical crosslinking of the composite via a hydrogen abstracting free radical initiation process which has the potential to crosslink the composite in one step to enhance its mechanical properties and also may have the ability to covalently link proteins within its structure. Bovine serum albumin (BSA)[19], covalently bound to the structure of the composite, was used as a model protein in the current study.

Materials and Methods

CS (MW 100,000 to 300,000 g/mol) and acetic acid were supplied by Fisher Scientific (Acros Organics). Poly(ethylene glycol) dimethacrylate (PEGDMA) MW 200 and 600 g/mol were obtained from PolySciences Inc. Calcium chloride (CaCl_2), ammonium phosphate dibasic ($(\text{NH}_4)_2\text{HPO}_4$), ammonium hydroxide, sodium bicarbonate and benzophenone were purchased from Sigma-Aldrich. All materials were used as received.

Scaffold preparation

Hydroxyapatite (HAp) was prepared using a precipitation method adapted from Murugan & Ramakrishna [15] by Killion et al[20]. Briefly, HAp was prepared by a wet chemical method using CaCl_2 and $(\text{NH}_4)_2\text{HPO}_4$ as Ca and P precursors. An aqueous 0.3 M $(\text{NH}_4)_2\text{HPO}_4$ solution was added drop-wise to a 0.5 M CaCl_2 aqueous solution under continuous stirring (1000 rpm) at 60 °C for 1 h while maintaining the pH above 10 by adding ammonium hydroxide. The sample was left overnight to precipitate out of solution before decanting, filtering and washing several times with distilled water to remove any residual ammonium hydroxide. The sample was microwave irradiated for 10 min and the resultant material was vacuum dried at 50 mbar for 24 h at 60 °C. After 24 h, the material was ground with a mortar and pestle and sieved through a micro-filter with a pore size of 45 μm .

For sample preparation, various ratios of HAp and CS were dry mixed in a beaker. To this mixture a 1% acetic acid solution in distilled water was added to yield 20% w/v CS paste. This was thoroughly mixed until a homogenous paste was produced. Subsequently, PEGDMA was added to the paste and thoroughly mixed. Finally, a 0.1% w/v benzophenone solution in ethanol was added and the paste again thoroughly mixed. Fixed weight samples ($2 \text{ g} \pm 0.1 \text{ g}$ for compression/swelling studies; $200 \text{ mg} \pm 0.1 \text{ mg}$ for Fourier transform infrared spectroscopy (FTIR)/controlled release experiments) were then weighed and positioned into a silicone mould prior to placing under a UV curing system (Dr. Gröbel UV-Electronic GmbH). The irradiation chamber comprised a controlled radiation source with 20 UV tubes that provide a spectral range between 315 and 400 nm at an average intensity of 10–13.5 mW cm^2 . Samples were cured for 40 min turning the sample over mid-curing. Following curing, samples were submersed in a 0.1 M sodium bicarbonate solution for 10 min to neutralize the acidic solution. Control samples were placed into the mould and UV irradiated to ensure identical handling of all samples. Samples containing the model protein BSA were made in a similar fashion with BSA being added during the HAp/CS mixing process.

Surface morphological images of representative scaffolds were taken using a Mira scanning electron microscope (SEM) in back-scattered electron (BSE) mode. Prior to imaging,

samples were placed on an aluminium stub and sputter coated with a gold using Baltec SCD 005 for 110 s at 0.1 mBar vacuum before testing.

Fourier transform infrared spectroscopy

Attenuated total reflectance FTIR was carried out on a Perkin-Elmer Spectrum One fitted with a universal ATR sampling accessory. Tests were run in the spectral range of 4000 – 650 cm^{-1} , utilising a 4 scan per sample cycle with a resolution of 0.5 cm^{-1} at ambient temperature. Prior to commencing data collection, an operator experienced in FTIR ensured that a suitable spectrum was visible with minimal noise during the set-up phase of the test.

In vitro stability

In vitro stability analysis was performed in pH 7.4 phosphate buffered saline (PBS) at 37 °C. Test specimens consisted of CS and various ratios of CS/HAp chemically crosslinked with PEG200DMA. During testing neutralized samples (n=3) were submerged in PBS. Periodically samples were removed, their integrity assessed during handling and their pat dried weights recorded. This was continued for 10 weeks or until the samples disintegrated during handling.

Compression testing

Samples for compression testing (Table 1) were placed into pH 7.4 PBS for 1 h prior to testing. Samples (n=6) were tested with a Lloyd LRX tensometer using a 2.5 KN load cell in compression mode. Sample dimensions were input individually into the control software and had a range of $2.0 \pm 0.2 \text{ mm} \times 21 \pm 0.6 \text{ mm}$ diameter. Samples were strained at a rate of 0.5 mm/min to a displacement of 60 % [20].

Swelling

The swelling characteristics of CS and CS: HAp composites (Table 2; n=3) crosslinked with PEG200DMA and PEG600DMA moieties were assessed by drying UV irradiated samples in a vacuum oven at 37 °C, 50 mm Hg for 120 h to obtain the sample dry weight. Samples were then placed in pH 7.4 PBS at ambient temperature for 48 h at which point the samples had reached swollen equilibrium. Subsequently, samples were again dried in a vacuum oven at 37 °C, 50 mm Hg until a constant weight was observed. Using these data the equilibrium water content (EWC), percentage swelling, water uptake (Wu) and gel fraction were calculated as follows:

$$\text{EWC} = 100(W_t - W_0)/W_t \quad [21] \quad \text{Eq1}$$

$$\text{Wu} = 100(W_t - W_0)/W_0 \quad [21] \quad \text{Eq2}$$

$$\% \text{ Swelling} = (W_t - W_0)/W_0 \times 100 \quad [21] \quad \text{Eq3}$$

$$\text{Gel fraction} = \frac{W_d}{W_0} \times 100 \quad \text{poly(ethylene glycol) dimethacrylate [22]} \quad \text{Eq4}$$

Where: W_t represents the dry weight of the sample following swelling.

To assess the efficacy of the crosslinking reaction, the dried samples as detailed above were submerged in a 1% acetic acid solution for 48 h, following which remaining samples were again dried and the gel fraction recalculated using the final dried weight (W_{df})

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was carried out using a TA Instruments 2920 DSC with an inline refrigeration unit. Composite samples weighing between 8 and 10 mg were measured using a Sartorius scale at a resolution of 1×10^{-5} g. Samples were initially heated from ambient temperature to 160 °C at a rate of 20 °C per min to remove moisture and any potential thermal history. The samples were maintained at 160 °C for 2 min and cooled to 50 °C at 20 °C before finally being heated at a rate of 10 °C per min until they reached 350 °C. All measurements were conducted in open aluminum pans using an empty aluminum pan as a reference. Tests were conducted under a constant flow of nitrogen at a rate of 30 ml/min. Calibration of the instrument was performed using indium as standard. The analysis was conducted on individual samples and the glass transition temperature (T_g), if present, was recorded. Tests were carried out in duplicate; however, a third run was performed if necessary to confirm reproducibility.

Cytocompatibility

The assessment of scaffold cytocompatibility was performed according to principles outlined in ISO 10993-5 where cell viability was calculated relative to cell viability following incubation on tissue culture plastic as the negative (non-toxic) control using the indirect contact method with Cell Titer-Blue® Assay as a toxicity endpoint. This method is based on the ability of viable cells to reduce resazurin (indicator dye) into highly fluorescent resorufin, and is thus a measure of metabolic activity[23].

MC3T3-E1 cells (American Type Culture Collection) were seeded at a density of 50,000 cells per well in 24-well plates and cultured to confluence. Culture media consisted of DMEM, 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma Aldrich). Scaffolds which had previously been washed in sterile water were also placed into separate 24-well plates with 1 ml of medium and again incubated for 24 h. After 72 h incubation, the media were removed from wells containing cells and discarded. These media were replaced with media which had been incubated with scaffolds for 24 h, and incubation continued for a further 24 h. At this time the media were aspirated and the cells washed with 0.1M PBS solution. After rinsing, 350 µl of fresh medium containing a 10% solution of Cell Titer-Blue® were added to the wells. Three no-cell control wells were included. Culture plates were wrapped in aluminum foil and incubated at 37 °C in an atmosphere of 5 % CO_2 and 95 % humidity for 4 h. After incubation, a 150 µl aliquot of each sample and media with dye alone, were transferred to a white opaque 96-well plate (Falcon® Becton Dickinson Labware, France) in duplicate. Subsequently, the fluorescence values of samples were

measured (excitation/emission: 560/590 nm) using a Multilabel Counter Wallac 1420 (Perkin Elmer, US). The results were calculated by averaging fluorescent values of the samples and subtracting average value of for media only. Cell viability was presented as a percentage of tissue culture plastic controls.

Bovine Serum Albumin Enzyme-Linked Immunosorbent Assay

BSA [24] was used as a model protein to assess protein retention and release. The release rate of BSA from the scaffolds was assessed by placing scaffolds containing BSA into 24-well plates. Subsequently, 1 ml of sterile PBS was added to each well and the plate was incubated at 37 °C for predetermined time intervals from 1 h to 10 d. At each timepoint the PBS was removed and replaced with fresh PBS. The removed aliquots were frozen until testing.

CS/HAp controls without BSA were included to exclude the possibility that either component interfered with the enzyme-linked immunosorbent assay (ELISA) readings. The amount of BSA released at each timepoint was assessed using a commercially available ELISA kit (USCN Life Sciences Inc) according to the manufacturer's instructions. The optical density of all samples was read using a plate reader at 450 nm. Concentrations of BSA in test samples were calculated by interpolation using a standard curve. Scaffolds were tested in triplicate with the mean of two replicates taken as the absorbance value for the amount of BSA released.

Results

Samples retained the shape of the mould following curing. SEM photomicrographs indicate that the HAp particle size ranged from the nano to microscale. Agglomerates of HAp which were trapped in the CS matrix were observed as large bright areas of the photomicrograph (Figure 1). Neutralized CS controls and CS/HAp crosslinked composites (n=3) were placed into PBS pH 7.4 at 37 °C to determine their *in vitro* degradation profile. From the analysis of the relative weight of the samples, it was found that CS alone remained intact for up to 45 d. However, at 51 d while the samples retained their shape, they had lost their mechanical integrity and fell apart during handling prior to weighing. Therefore no measurement was possible. Similarly, one composite containing a CS:HAp ratio of 1:4 began to breakdown at 45 d, while all three samples disintegrated during handling after 10 weeks in PBS. However, samples containing CS: HAp ratios of both 1:1 and 1:2 retained their shape and integrity after 10 weeks in PBS (Figure 2).

CS is characterised by resonance bands at 1154 and 893 cm^{-1} identifying its saccharide groups. Other bands at 1600, 1651 and 3425 cm^{-1} depict N-H bending of the primary amino groups, the carbonyl stretching of the amide bands, and the N-H stretching of the primary amino groups, respectively [25]. In the current study the peak associated with the saccharide groups was observed at 1150 cm^{-1} in the spectrum of the control CS and CS-HAp 1:1 samples; however, the peak at 893 cm^{-1} was not detected (Figure 3). Separate peaks were not detected in the region of 1600 to 1650 cm^{-1} ; however a peak was observed in the region of 1638 cm^{-1} . A broad peak was observed between 3327 and 3348 cm^{-1} representing N-H stretching of the primary amino groups and the presence of water. For crosslinked CS

samples the peak representative of the saccharide groups had shifted to $1165 - 1186 \text{ cm}^{-1}$ for crosslinked samples.

The main characteristic peaks of PEGDMA have been reported to be due to OH stretching at 3570 cm^{-1} , CH stretching at 2870 cm^{-1} , C=C bending at 1720 and 1640 cm^{-1} and CCH₂ bending at 1450 cm^{-1} [20]. Of particular relevance to the current study, no peaks associated with C=C bending were observed following UV curing for CS, PEG600DMA; or CS: HAp 1:1, PEG600DMA, whereas, for CS: HAp 1:1, PEG200DMA; a shoulder was observed on the 1638 cm^{-1} peak while for the CS, PEG200DMA a small peak was visible, both in the region of 1710 cm^{-1} .

In previous work, Killion et al [20] described the characteristic peaks of the HAp employed in the current study. The most notable peaks in the region analysed were at 1023 cm^{-1} (corresponding to PO₃/4, ν_3 stretching) and 962 cm^{-1} (associated with PO₃/4, ν_1 stretching). In the current study, these peaks were observed at $1030-1031 \text{ cm}^{-1}$ and 962 cm^{-1} respectively. However the intensity of the peak at 962 cm^{-1} was greatly reduced compared to that reported in previous work [20].

In the current study there was no evidence of characteristic FTIR peaks for BSA associated with 3430 , 3062 , 1652 , and 1531 cm^{-1} which, are assigned to the stretching vibration of –OH, amide A (mainly –NH stretching vibration), amide I (mainly C=O stretching vibrations), and amide II (the coupling of bending vibration of N–H and stretching vibration of C–N) bands, respectively [26].

The mechanical properties of different composites were assessed using compression testing following submersion in PBS for 1 h. Young's modulus (Figure 4) values varied from 2.8 MPa for CS controls to 3.8 MPa for crosslinked CS, and 4.7 and 4.8 MPa for CS: HAp composites at ratios of 1:1 and 1:2 respectively. From statistical analysis of Young's modulus it was found that CS: HAp in ratios of 1:1 and 1:2 had a significantly higher Young's modulus than CS alone ($p = 0.019$ for both comparisons). No further significant differences were observed ($p = 0.329$ for all comparison).

EWC, percent swelling, water uptake and gel fraction measurements are shown in Table 3. The incorporation of HAp resulted in a significant decrease in EWC, percent swelling and water uptake of the scaffold ($p = 0.026$ for all comparisons). The incorporation of the crosslinking agent at either molecular weight had no effect on these parameters ($p = 0.756$ for all comparisons). CS composites crosslinked with PEG600DMA had a significantly lower gel fraction than all other samples ($p = 0.026$ for all comparisons). No further statistical differences were detected ($p = 0.053$).

Following swelling in acetic acid solution only CS composites crosslinked with PEG600DMA, and CS/HAp crosslinked composites did not dissolve. Hence, these scaffolds had significantly higher gel fractions after swelling in acetic acid solution than the other scaffolds tested ($p < 0.001$). No significant differences were detected between the CS/HAp crosslinked scaffolds ($p = 1$). However, both CS/HAp crosslinked scaffolds had a statistically higher gel fraction than CS crosslinked with PEG600DMA.

Differential Scanning Calorimetry

DSC thermograms indicated a weak T_g in some of the samples tested which varied from 119 to 234 °C depending on the composition of the scaffolds (Figure 5). CS control samples and CS PEG200DMA exhibited T_gs in the region of 146.8±1.9 °C and 143.6 °C respectively, although a T_g value was only observed for 1 of 2 CS PEG200DMA test samples. One of three CS, PEG600DMA test samples exhibited a T_g at 119.3 °C whereas no baseline shift was observed in the other two repeated runs. Similarly, no base line shift was observed for CS: HAp 1:1. However, the T_g values for CS: HAp 1:1, PEG200DMA and CS: HAp 1:1, PEG600DMA samples were 213.28 and 234.5 °C respectively (1 of two replicates exhibited a T_g in both instances).

CS control samples, CS PEG200DMA and CS PEG600DMA scaffolds exhibited exothermic transitions at 297.9±2.5, 296.9±0.6 and 298.8±1.7 °C respectively, whereas, CS/HAp control samples, CS/HAp PEG200DMA and CS/HAp PEG600DMA scaffolds exhibited exothermic transitions at 305.1±0.3, 306.4±0.9 and 302.7±1.6 °C respectively. Samples turned black after testing.

Cytocompatibility

Cells exposed to leachates from the crosslinked scaffolds retained full viability relative to tissue culture plastic (data not shown).

Bovine Serum Albumin ELISA

ELISA was used to determine the amount of BSA released over time. CS PEG200DMA control samples were used to confirm that the ELISA did not yield a response to the construct without BSA. When 400 µg albumin were added to a total sample size of 200 mg, CS PEG200DMA samples release approximately 25 % of the BSA loading during a ten day period. This release was relatively linear. The release rate of BSA increased with both the incorporation of HAp and with increases in the molecular weight of the crosslinking agent to 75% and 100 % of the BSA loading respectively, during the ten day observation period (Figure 6). CS/HAp PEG200DMA appeared to have a burst release phase in the first 24 hours where ca 12% of the BSA loading was released, was followed by linear release up to ten days. CS/HAp PEG600DMA had a distinct burst release phase where 45% of the BSA loading was released after one day and 70% released after three days, followed by sustained release up to the the experiment endpoint.

Discussion

The aim of this study was to explore the possibility of developing an osteomimetic scaffold with the appropriate Mechanical properties, and to which proteins could be covalently attached for delivery to osseous defects. Ideally, the scaffold should degrade at the same rate as repair osteogenesis proceeds, thereby ensuring a progressive replacement of the implant with new bone[27]. Based on these design criteria, we focused on a composite scaffold formed from HAp and CS.

CS was selected as it is of natural origin, abundant, inexpensive, biocompatible, and has antimicrobial properties [28]. Importantly, it has been reported that CS biodegrades in a timeframe between 2–8 weeks[29], which seems appropriate for the application discussed here. In the current study, CS was found to breakdown in PBS after approximately 6 weeks. However, this was using static, *in vitro* conditions. It is reasonable to assume that if this scaffold were to be implanted into a dynamic *in vivo* environment, the degradation rate would be considerably faster. However, with the addition of HAp at ratios of 1:1 and 1:2 the scaffold retained its shape and structural integrity throughout the 10 week test period. This is likely to be due to physical bonding between HAp and the polymeric component of the composite, as was observed in previous work[20].

The polarity of the HAp may also have increased the efficacy of the chemical crosslinking reaction. This was observed in previous work involving PEGDMA crosslinking of Polyvinylpyrrolidone where it was found that the incorporation of polar acrylic acid increased the efficiency of the reaction[21]. Composites containing a CS/HAp ratio of 1:4 broke down mechanically between 6 and 10 weeks. The ability of the reinforcing agent to increase the stability and strength of the polymer matrix in a composite structure is dependent on intimate contact between the individual components[30]. In the current study the reduction in stability for CS/HAp composites at a ratio of 1:4 can be attributed to the insufficient polymer matrix to ‘wet’ completely the ceramic material. This would result in voids between the individual components that, in turn, reduced the stability of the scaffold.

FTIR analysis confirmed the presence CS and HAp in the composite. Additionally the absence of a peak associated with C=C for CS, PEG600DMA and CS:HAp 1:1, PEG600DMA scaffolds indicates that the crosslinking reaction was successful. The presence of the shoulder/peak at 1710 cm^{-1} for CS:HAp 1:1, PEG200DMA and CS, PEG200DMA samples respectively may be due to the bulky nature of the CS repeat unit which prevented the relatively short chain crosslinking agent successfully reacting to create a crosslink. This could have resulted in branch formation rather than crosslinks [21]. The intensity of the peak associated with PO₃/4, ν₁ stretching for HAp was found to be greatly reduced in the current study compared to previous work[20] where similar concentrations of HAp were utilised. This would indicate that intramolecular bonding occurred with this group, which increased the stability of the entire construct. No evidence was found of BSA in the FTIR spectrum of composite samples prior to and post crosslinking due to the low relatively low concentration of BSA within the system. Hence, it is not possible to verify whether BSA was crosslinked within the system or was merely entrapped within the composite structure. However, due to the method of crosslinking employed, it is still highly likely that BSA was crosslinked within the structure during the free radical reaction process.

The mechanical stability of a fracture repair is known to affect tissue formation and vascularity[31]. The load bearing capacity of a scaffold may also influence the healing properties of an engineered construct. Hence, it was important to maximize the load bearing capacity of the cross-linked scaffold. This was done using a ‘design of experiments’ approach to determine appropriate levels of photoinitiator and crosslinking agent. Having defined these parameters different combinations of CS and HAp were assessed. From compression testing, it was found that crosslinking the structure and the addition of HAp to

CS increased the mechanical properties to 5MPa from less than 3MPa for CS controls. These values are fall short of those for cortical bone and cancellous bone (Young's modulus 14–20 GPa and 50–500 MPa respectively[15]. However, results from the current study compare favorably to reports in the literature for hydrogel/ceramic composites. For instance Gleeson et al, [32]reported that collagen/HAp crosslinked composites had Young's modulus at 10 % strain of 1.3 KPa which is several orders of magnitude lower, whereas Killion et al [20,33] reported values of between 4 and 12MPa at 60 % strain for PEGDMA ceramic based composites. While the values reported by Killion et al [22,33] are higher than those reported here, the matrix used by the latter authors was a highly crosslinked, non-biodegradable polymer unlike the biodegradable polymer system reported in the current study. As with stability, the mechanical properties of the composite are dependent on intimate contact between the polymer and matrix but, as Gleeson et al [32] reported, these properties can be further enhanced by crosslinking the structure. Nevertheless, it is envisaged that the proposed scaffold would be used in conjunction with a fixation device and as such, the initial compressive strengths reported here would not necessarily limit its clinical application.

The swelling and gel fraction of the scaffolds was found to be dependent on HAp content and molecular weight of the crosslinking agent. The EWC of CS based scaffolds was between 51.7 and 53.1 %. Unsurprisingly, the addition of HAp to the composite reduced this, as the HAp component did not absorb large volumes of water. Similarly, Wu and percent swelling were also affected by HAp addition. The gel fraction in PBS ranged from 94 % to 100 % for all samples except CS, PEG600DMA that had a significantly lower gel fraction in PBS of 88 %. The latter scaffolds had a large volume by weight of the water soluble PEG600DMA to maintain stoichiometric ratios. The low gel fraction recorded indicates that not all the crosslinking agent had reacted and that unreacted crosslinking agent washed from the scaffold. Nevertheless, when HAp was added to the scaffold the enhanced polarity of the scaffold due to the presence of HAp increased the efficiency of the crosslinking reaction and the corresponding scaffold containing HAp had a significantly higher gel fraction. The subsequent swelling in an acetic acidic solution, led to scaffolds which were not successfully crosslinked completely dissolving. Hence, both samples without crosslinking agent completely dissolved, indicating the success of the UV initiated crosslinking reaction. However, CS, PEG200DMA crosslinked scaffolds also dissolved whereas the remaining samples had a gel fraction of between 42 and 62 %. These findings are in agreement with FTIR analysis which indicated that the crosslinking reaction was not successful for CS, PEG200DMA crosslinked scaffolds as indicated by the presence of the C=C bond in the IR spectrum following UV curing. The significant increase in gel fraction of crosslinked scaffolds incorporating HAp further indicates that its incorporation increased the efficacy of the crosslinking reaction.

In thermal analysis of CS, moisture can act as a plasticizer and thus should be removed prior to testing. However, it is advisable to avoid the degradation temperature of CS, which has been reported to be between 200–250 °C [34], when making the first heating run. Hence, in the current study, the initial heating run for CS was stopped at 160 °C. On the subsequent heating run an exothermic peak was observed between 297 and 299° C for scaffolds containing CS only. Upon visual observation following testing, the scaffolds had turned black which indicates that this transition is a result of degradation. Such findings are

reflected in the literature where Martínez-Camacho et al. [34] have reported that the degradation Temperature (Td) of CS is 285 °C whereas Ferreira et al. [35] reported the Td to be 300° C. With the incorporation of HAp the Td of the scaffold increased to between 303 and 307 °C. This correlates with FTIR and gel fraction measurements which indicate an increase in intermolecular bonding and crosslinking efficiency with the incorporation of HAp. Similar findings were reported by Venkatesat el al [36] who hypothesized that slight increases in the degradation temperature of CS based composites were a result of covalent bonding between CS and the reinforcing filler.

The Tg of a polymer is a measure of the rigidity of the polymer backbone chain. For CS there is a range of reported values for Tg. Ferreira et al. [35] reported that the Tg of CS is 131° C and that a transition at 200 °C, while often referred as a Tg, is more likely a liquid-liquid interaction which corresponds to motion of very long segments or even the whole polymer chain. Conversely, Martínez-Camacho et al. [34] report that pure CS has presented values of 203 °C and 104 °C. On the other hand, Dhawade & Jagtap [37] found that the Tg of dried CS is 118 °C which reduces to 61 °C if water is present confirming that water acts as a plasticizer. In the current study, samples were firstly dried hence, water could not affect the calculation of Tg. However, it was not always possible to observe a clear baseline shift, which is indicative of a Tg. Nevertheless, when the transition was observed it was in the region of 119 to 147 °C for CS based samples, which moved to 213 to 234 °C for CS/HAp scaffolds. This increase in Tg was also observed by Li *et al.* (2010) [38] who found that the incorporation of HAp increased the Tg values of the composite in relation to the base polymers. In the current study the crosslinking of the composite likely also effected Tg calculations as highly crosslinked structures do not have a flexible polymer backbone and as such do not exhibit a Tg. This could account for the failure to detect a Tg in some of the crosslinked samples.

CS and HAp are widely reported as biocompatible alone and in combination [15,39–41]. In agreement with this, no cytotoxic response was observed for any of the composites tested in the current study.

BSA release began within hours of the scaffold being placed into PBS. This sustained release could be a result of unbound and poorly bound BSA slowly leaching from the scaffold initially, with covalently linked BSA released following a number of days. The rate of BSA release increased with the incorporation of HAp into the CS system. This may be due to HAp taking up physical space between the polymer chains thereby interfering with the crosslinking reaction. The release of BSA also increased with an increase in the molecular weight of the crosslinking agent. These findings were also reported in previous work where longer crosslink chains enabled easier diffusion of the incorporated active pharmaceutical ingredient [42,43]. The BSA release rates found in the current study compare favorably with those reported in the literature using alternative scaffolds. Boonsongrit et al. [44] examined BSA release from HAp microspheres and poly(lactic-glycolic acid) (PLGA) encapsulated HAp microspheres. BSA was incorporated into the system using an absorption method which led to burst BSA release from the HAp microspheres, with up to 80 % of the cargo BSA released within 2 h. This release rate was extended to 9 d for approximately 70% release when microspheres were incorporated into

the PLGA matrix. However, the PLGA matrix is slowly degraded and would remain in the bone defect longer than required. Yang et al. [45] also examined BSA release from PLGA microspheres. In this instance approximately 70% of the BSA was released by 2 d incubation at 38 °C; minimal further BSA release occurred during the subsequent 16 d. Another study investigating the effect of preparation temperature on BSA release from PLGA microspheres illustrated that at all temperatures an initial burst release was observed which accounted for between 20 and 60% of the protein payload within 24 hours, with minimal release of BSA thereafter [45]. These results correlate with current findings where HAP incorporation into the scaffold increased observed BSA release rates.

In conclusion, we have developed a biocompatible, mechanically robust scaffold with an appropriate stability profile for bone healing. Proteins can be covalently attached to the scaffold, from which they are released for an extended period under *in vitro* conditions. Further research is required to determine its ability to deliver osteogenic factors and repair osseous lesions *in vivo*.

Acknowledgments

This study was supported in part by AIT's President Seed fund, a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme (NanoFact 298107,) and the National Institutes of Health (NIAMS RO1 AR050243).

References

1. Evans CH. Advances in regenerative orthopedics. *Mayo Clin. Proc.* 2013; 88:1323–1329. DOI: 10.1016/j.mayocp.2013.04.027 [PubMed: 24182709]
2. Little N, Rogers B, Flannery M. Bone formation, remodelling and healing. *Surgery.* 2011; 29:141–145. DOI: 10.1016/j.mpsur.2011.01.002
3. McKibbin B. The biology of fracture healing in long bones. *J. Bone Joint Surg. Br.* 1978; 60-B: 150–162. DOI: 10.1007/978-1-4471-5451-8_139
4. Papakostidis C, NK K, Pretel J, Faour O, DJ M, PV G. Prevalence of complications of open tibial shaft fractures stratified as per the. *Am J Orthop.* 2011; 40:450–451. [PubMed: 22022673]
5. Verrier S, Alini M, Alsberg E. Tissue Engineering and Regenerative Approaches to Improving the Healing of Large bone Defects. *Eur Cells Mater.* 2016 in press.
6. Calori GM, Mazza E, Colombo M, Ripamonti C. The use of bone-graft substitutes in large bone defects: Any specific needs? *Injury.* 2011; 42doi: 10.1016/j.injury.2011.06.011
7. Cancedda R, Giannoni P, Mastrogiacomo M. A tissue engineering approach to bone repair in large animal models and in clinical practice. *Biomaterials.* 2007; 28:4240–4250. doi:S0142-9612(07)00462-0 (pii)r. DOI: 10.1016/j.biomaterials.2007.06.023 [PubMed: 17644173]
8. Nyström E, Ahlqvist J, Legrell PE, Kahnberg KE. Bone graft remodelling and implant success rate in the treatment of the severely resorbed maxilla: a 5-year longitudinal study. *Int. J. Oral Maxillofac. Surg.* 2002; 31:158–164. DOI: 10.1054/ijom.2001.0197 [PubMed: 12102413]
9. Faour O, Dimitriou R, Cousins Ca, Giannoudis PV. The use of bone graft substitutes in large cancellous voids: Any specific needs? *Injury.* 2011; 42doi: 10.1016/j.injury.2011.06.020
10. Evans CH, Palmer GD, Pascher A, Porter R, Kwong FN, Gouze E, Gouze J-N, Liu F, Steinert A, Betz O, Betz V, Vrahas M, Ghivizzani SC. Facilitated endogenous repair: making tissue engineering simple, practical, and economical. *Tissue Eng.* 2007; 13:1987–93. DOI: 10.1089/ten.2006.0302 [PubMed: 17518747]
11. Lieberman JR, Daluiski A, Einhorn Ta. The Role of Growth Factors in the Repair of Bone. *J. Bone Jt. Surgery.* 2002; 84:1032–1044.

12. Carragee EJ, Hurwitz EL, Weiner BK. A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. *Spine J.* 2011; 11:471–491. DOI: 10.1016/j.spinee.2011.04.023 [PubMed: 21729796]
13. Carragee EJ, Ghanayem AJ, Weiner BK, Rothman DJ, Bono CM. A challenge to integrity in spine publications: Years of living dangerously with the promotion of bone growth factors. *Spine J.* 2011; 11:463–468. DOI: 10.1016/j.spinee.2011.06.001 [PubMed: 21729794]
14. King WJ, Krebsbach PH. Growth factor delivery: How surface interactions modulate release in vitro and in vivo. *Adv. Drug Deliv. Rev.* 2012; 64:1239–1256. DOI: 10.1016/j.addr.2012.03.004 [PubMed: 22433783]
15. Murugan R, Ramakrishna S. Development of nanocomposites for bone grafting. *Compos. Sci. Technol.* 2005; 65:2385–2406. DOI: 10.1016/j.compscitech.2005.07.022
16. Venkatesan J, Kim SK. Nano-hydroxyapatite composite biomaterials for bone tissue engineering - A review. *J. Biomed. Nanotechnol.* 2014; 10:3124–3140. DOI: 10.1166/jbn.2014.1893 [PubMed: 25992432]
17. Romagnoli C, D'Asta F, Brandi ML. Drug delivery using composite scaffolds in the context of bone tissue engineering. *Clin. Cases Miner. Bone Metab.* 2013; 10:155–161. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3917575&tool=pmcentrez&rendertype=abstract>. [PubMed: 24554923]
18. Murugan R, Ramakrishna S. SCIENCE AND Development of nanocomposites for bone grafting. 2005; 65:2385–2406. DOI: 10.1016/j.compscitech.2005.07.022
19. Uludag H, Yang J. Targeting systemically administered proteins to bone by bisphosphonate conjugation. *Biotechnol. Prog.* 2002; 18:604–611. DOI: 10.1021/bp0200447 [PubMed: 12052079]
20. Killion JA, Geever LM, Devine DM, Higginbotham CL. Fabrication and in vitro biological evaluation of photopolymerisable hydroxyapatite hydrogel composites for bone regeneration. *J. Biomater. Appl.* 2014; 28:1274–83. DOI: 10.1177/0885328213506951 [PubMed: 24114559]
21. Devine DM, Higginbotham CL. Synthesis and characterisation of chemically crosslinked N-vinyl pyrrolidinone (NVP) based hydrogels. *Eur. Polym. J.* 2005; 41:1272–1279. DOI: 10.1016/j.eurpolymj.2004.12.022
22. Killion JA, Geever LM, Devine DM, Kennedy JE, Higginbotham CL. Mechanical properties and thermal behaviour of PEGDMA hydrogels for potential bone regeneration application. *J. Mech. Behav. Biomed. Mater.* 2011; doi: 10.1016/j.jmbbm.2011.04.004
23. Czekanska EM, Stoddart MJ, Richards RG, Hayes JS. In search of an osteoblast cell model for in vitro research. *Eur. Cells Mater.* 2012; 24:1–17.
24. Quaglia F. Bioinspired tissue engineering: The great promise of protein delivery technologies. *Int. J. Pharm.* 2008; 364:281–297. DOI: 10.1016/j.ijpharm.2008.04.030 [PubMed: 18538517]
25. Homayoni H, Ravandi SaH, Valizadeh M. Electrospinning of chitosan nanofibers: Processing optimization. *Carbohydr. Polym.* 2009; 77:656–661. DOI: 10.1016/j.carbpol.2009.02.008
26. Huang P, Li Z, Hu H, Cui D. Synthesis and Characterization of Bovine Serum Albumin-Conjugated Copper Sulfide Nanocomposites. *J. Nanomater.* 2010; 2010:1–6. DOI: 10.1155/2010/641545
27. Calori GM, Giannoudis PV. Enhancement of fracture healing with the diamond concept: The role of the biological chamber. *Injury.* 2011; 42:1191–1193. DOI: 10.1016/j.injury.2011.04.016 [PubMed: 21596376]
28. Pérez, Ra, Won, JE., Knowles, JC., Kim, HW. Naturally and synthetic smart composite biomaterials for tissue regeneration. *Adv. Drug Deliv. Rev.* 2013; 65:471–496. DOI: 10.1016/j.addr.2012.03.009 [PubMed: 22465488]
29. Azab AK, Orkin B, Doviner V, Nissan A, Klein M, Srebnik M, Rubinstein A. Crosslinked chitosan implants as potential degradable devices for brachytherapy: In vitro and in vivo analysis. *J. Control. Release.* 2006; 111:281–289. DOI: 10.1016/j.jconrel.2005.12.014 [PubMed: 16499987]
30. Chen Y, Geever LM, Killion JA, Lyons JG, Higginbotham CL, Devine DM. Halloysite nanotube reinforced polylactic acid composite. *Polym. Compos.* 2015; n/a-n/a. doi: 10.1002/pc.23794
31. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. *BMC Med.* 2011; 9:66.doi: 10.1186/1741-7015-9-66 [PubMed: 21627784]

32. Gleeson JP, Plunkett Na, O'Brien FJ. Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration. *Eur. Cells Mater.* 2010; 20:218–230. doi:vol020a18 (pii).
33. Killion JA, Kehoe S, Geever LM, Devine DM, Sheehan E, Boyd D, Higginbotham CL. Hydrogel/bioactive glass composites for bone regeneration applications: synthesis and characterisation. *Mater. Sci. Eng. C. Mater. Biol. Appl.* 2013; 33:4203–4212. DOI: 10.1016/j.msec.2013.06.013 [PubMed: 23910334]
34. Martínez-Camacho, aP, Cortez-Rocha, MO., Ezquerro-Brauer, JM., Graciano-Verdugo, aZ, Rodríguez-Félix, F., Castillo-Ortega, MM., Yépiz-Gómez, MS., Plascencia-Jatomea, M. Chitosan composite films: Thermal, structural, mechanical and antifungal properties. *Carbohydr. Polym.* 2010; 82:305–315. DOI: 10.1016/j.carbpol.2010.04.069
35. Ferreira P, Coelho JFJ, dos Santos KSCR, Ferreira EI, Gil MH. Thermal Characterization of Chitosan- Grafted Membranes to be Used as Wound Dressings. *J. Carbohydr. Chem.* 2006; 25:233–251. DOI: 10.1080/07328300600734085
36. Venkatesan J, Qian Z-J, Ryu B, Ashok Kumar N, Kim S-K. Preparation and characterization of carbon nanotube-grafted-chitosan – Natural hydroxyapatite composite for bone tissue engineering. *Carbohydr. Polym.* 2011; 83:569–577. DOI: 10.1016/j.carbpol.2010.08.019
37. Dhawade P, Jagtap R. Characterization of the glass transition temperature of chitosan and its oligomers by temperature modulated differential scanning calorimetry. *Adv. Appl. Sci.* 2012; 3:1372–1382. doi:0976-8610.
38. Li J, Zhu D, Yin J, Liu Y, Yao F, Yao K. Formation of nano-hydroxyapatite crystal in situ in chitosan-pectin polyelectrolyte complex network. *Mater. Sci. Eng. C.* 2010; 30:795–803. DOI: 10.1016/j.msec.2010.03.011
39. Puppi D, Chiellini F, Piras aM, Chiellini E. Polymeric materials for bone and cartilage repair. *Prog. Polym. Sci.* 2010; 35:403–440. DOI: 10.1016/j.progpolymsci.2010.01.006
40. Liu C, Xia Z, Czernuszka JT. Design and Development of Three-Dimensional Scaffolds for Tissue Engineering. *Chem. Eng. Res. Des.* 2007; 85:1051–1064. DOI: 10.1205/cherd06196
41. Correlo VM, Boesel LF, Bhattacharya M, Mano JF, Neves NM, Reis RL. Hydroxyapatite reinforced chitosan and polyester blends for biomedical applications. *Macromol. Mater. Eng.* 2005; 290:1157–1165. DOI: 10.1002/mame.200500163
42. Devine DM, Devery SM, Lyons JG, Geever LM, Kennedy JE, Higginbotham CL. Multifunctional polyvinylpyrrolidinone-polyacrylic acid copolymer hydrogels for biomedical applications. *Int. J. Pharm.* 2006; 326:50–59. DOI: 10.1016/j.ijpharm.2006.07.008 [PubMed: 16926073]
43. Devine DM, Geever LM, Higginbotham CL. Drug release from a N-vinylpyrrolidinone/acrylic acid lubricious hydrophilic coating. *J. Mater. Sci.* 2005; 40:3429–3436. DOI: 10.1007/s10853-005-0416-2
44. Boonsongrit Y, Abe H, Sato K, Naito M, Yoshimura M, Ichikawa H, Fukumori Y. Controlled release of bovine serum albumin from hydroxyapatite microspheres for protein delivery system. *Mater. Sci. Eng. B.* 2008; 148:162–165. DOI: 10.1016/j.mseb.2007.09.006
45. Yang YY, Chia HH, Chung TS. Effect of preparation temperature on the characteristics and release profiles of PLGA microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. *J. Control. Release.* 2000; 69:81–96. DOI: 10.1016/S0168-3659(00)00291-1 [PubMed: 11018548]

Highlights

biocompatible, mechanically robust scaffold with an appropriate stability profile for bone healing when used with fixation device

Proteins can be covalently attached to the scaffold

Proteins are released for an extended period under *in vitro* conditions

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Photomicrograph of HAp/CS 1:1 PEG600DMA scaffold

HAp was observed as bright sections of BSE photomicrographs. HAp particles embedded in the CS matrix were observed to be microscaled agglomerates.

Figure 2. Scaffold degradation in pH7.4 PBS at 30 °C (n = 3)

Samples comprising CS:HAp 1:1 and 1:2 retained their mechanical integrity for over 10 weeks under static conditions

Figure 3. FTIR Spectrum of CS based scaffolds

FTIR analysis indicates the presence of CS and HAp in the scaffolds. The peak associated with C=C is not visible for samples crosslinked with PEG600DMA indicating that crosslinking was successful.

Figure 4. Compressive Modulus of scaffolds (n = 6). Error bars represent standard deviation
The scaffold containing CS:HAp at a ratio of 1:1 or 1:2 had a Young's modulus that was significantly greater than that of chitosan alone. # indicate significant differences ($p < 0.05$).

Figure 5. Differential Scanning Calorimetry thermogram of a) CS control; b) CSPEG200DMA; c) CS PEG600DMA; d) CS:HAP 1:1 control; e) CS:HAP 1:1, PEG200DMA and f) CS:HAP 1:1, PEG600DMA (n = 2)

The thermogram indicates a slight shift in baseline indicating a T_g for some samples. All samples exhibited a sharp exothermic transition at ca 300 °C which coupled with blackening of the sample indicated thermal degradation.

Figure 6. The release of BSA from scaffolds during a ten day incubation. Error bars represent Standard Deviation

Scaffolds were tested in triplicate with the mean of two replicates taken as the absorbance value for the amount of BSA released.

Table 1
Composition of samples utilised for compression testing of crosslinked CS: HApsamples

Sample	CS (g)	HAp (g)	1% acetic acid solution (ml)	Bp Solution (µl)	PEG2000DMA (µl)
Crosslinked CS	2000	0	10	200	200
CS control	2000	0	10	0	0
CS:HAp 1:1	2000	2000	10	200	200
CS:HAp 2:1	2000	1000	10	200	200

CS: Chitosan; HAP: Hydroxyapatite; Bp: Benzophenone; PEG2000DMA: Poly(ethylene glycol) dimethacrylate, molecular weight 200

Table 2

Composition of samples utilised for swelling experiments

The volume of PEG600DMA was $3 \times$ that of PEG200DMA to maintain the same stoichiometric ratio

Sample	CS (g)	HAp (g)	1% acetic acid solution (ml)	Bp Solution (μl)	PEGDMA (μl)
CS control	200	0	1	0	0
CS, PEG200DMA	200	0	1	20	20
CS, PEG600DMA	200	0	1	20	60
CS: HAp 1:1	200	200	1	0	200
CS: HAp 1:1, PEG200DMA	200	200	1	20	200
CS: HAp 1:1, PEG600DMA	200	200	1	20	200

CS: Chitosan; HAp: Hydroxyapatite; Bp: Benzophenone; PEG200DMA: Poly(ethylene glycol) dimethacrylate, molecular weight 200; PEG600DMA: Poly(ethylene glycol) dimethacrylate, molecular weight 600

Table 3
EWC, percent swelling, water uptake and gel fraction measurements for CS and CS/HAp composites

Sample	EWC PBS $\pm$ SD	percent swelling $\pm$ SD	Water uptake $\pm$ SD	Gel fraction after PBS $\pm$ SD	Gel fraction after acetic acid sol $\pm$ SD
CS control	53.1 $\pm$ 0.2	213.2 $\pm$ 0.8	113.2 $\pm$ 0.8	97.3 $\pm$ 2.0	0.0 $\pm$ 0
CS, PEG200DMA	52.7 $\pm$ 1.0	211.3 $\pm$ 4.2	111.3 $\pm$ 4.2	96.2 $\pm$ 1.0	0.0 $\pm$ 0
CS, PEG600DMA	51.7 $\pm$ 2.0	207.2 $\pm$ 8.7	107.2 $\pm$ 8.7	88.2 $\pm$ 3.6	41.8 $\pm$ 5.2
CS:HAp 1:1	46.8 $\pm$ 1.4	188.1 $\pm$ 4.9	88.1 $\pm$ 4.9	100.2 $\pm$ 0.4	0.0 $\pm$ 0
CS:HAp 1:1, PEG200DMA	47.5 $\pm$ 1.7	190.3 $\pm$ 6.2	90.3 $\pm$ 6.2	96.5 $\pm$ 2.8	61.9 $\pm$ 1.3
CS:HAp 1:1, PEG600DMA	47.5 $\pm$ 1.2	190.7 $\pm$ 4.2	90.7 $\pm$ 4.2	94.2 $\pm$ 1.7	62.1 $\pm$ 4.7

CS: Chitosan; HAp: Hydroxyapatite; EWC: Equilibrium water content; PEG200DMA: Poly(ethylene glycol) dimethacrylate, molecular weight 200; PEG600DMA: Poly(ethylene glycol) dimethacrylate, molecular weight 600