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Development Of Synthetic Alternatives For Bone Tissue Engineering

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Abstract: Due to the inherent limitations of current biological bone grafts, alternative synthetic substitutes are being pursued. Therefore, this study aimed to improve the bioactive and compressive properties of photopolymerisable polyethylene glycol hydrogels with the incorporation of hydroxyapatite at different loadings. The synthesis of pure hydroxyapatite was verified through Fourier transform infrared spectroscopy (FTIR) analysis by the complete reaction of all constituents. X-ray diffraction confirmed a bioactive layer on the surface of the hydrogel based composites through the formation of carbonate hydroxyapatite. A reduction in percentage swelling and hydroxyapatite absorbing the compressive load resulted in the hydrogel composites with enhanced compression strength in terms of Young's modulus and storage modulus.

Key words: Hydrogels; photopolymerisation; hydroxyapatite; mechanical properties.

INTRODUCTION

Tissue engineering is the method of replicating tissue by using principles of engineering, medicine and physical sciences (Langer and Vacanti 1993). Recently, this area of research has received extensive investigation in the literature for bone regeneration (Kellomäki *et al*. 2000; Killion *et al*. 2011; Zhai *et al*. 2012). In 2006 it was estimated that 36 million Americans suffered from bone related diseases while 3 million were diagnosed with fracture related injuries. Bone grafting is a common surgical procedure; it has been estimated that 2.2 million grafting procedures are performed worldwide each year (Lewandrowski *et al*. 2000).

Hydrogels are a key group of biomaterials for tissue engineering (Geever *et al*. 2008; Ma *et al*. 2010). They have been utilised to support and assist in the restoration of a range of tissues, such as bone, cartilage, vessels and skin. Polyethylene glycol (PEG) based hydrogels have been widely investigated as a scaffold material for bone regeneration (Bryant and Anseth 2003; Gaharwar *et al*. 2011). However, they lack the mechanical strength for load bearing applications, in particular bone tissue engineering. Another type of material which have been used for bone grafts substitutes are bioceramics. They are biocompatible and have favourable osteoconductive and osteoinductive properties. The advantage of bioceramics over conventional polymers is their ability to form an apatite layer at the interface of the scaffold and surrounding host tissue (Raucci *et al*. 2010). Both hydrogels and bioceramics have different limitations in terms of mechanical properties which they need to overcome for bone regeneration application. To conquer these shortcomings, a new generation of polymeric and bioceramic composites have been developed. The mechanical properties of these composites can be modified to produce a synthetic bone graft substitute that contains toughness and plasticity from the polymeric phase and the bioactive properties of the bioceramics component.

The objectives of this paper were to synthesis and investigate the properties of polyethylene glycol and hydroxyapatite composites via photopolymerisation. The mechanical properties and hydrogel performance were characterised by synthesis, compression tests, gel fraction, percentage swelling and *in vitro* biomineralisation properties.

MATERIALS AND METHODS

Materials:

The macromolecular monomers, poly(ethylene glycol) dimethacrylate (*Mw*) 600 was obtained from PolySciences Inc. The photoinitiator utilised was 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure® 2959) supplied by Ciba Specialty Chemicals. All materials were used as received.

Hydroxyapatite Synthesis:

The hydroxyapatite (HAP) was prepared by wet chemical method using $CaCl_2$ and $(NH_4)_2HPO_4$ as Ca and P precursors which has been previously been prepared by Murugan *et al*. 2004 (Murugan and Ramakrishna 2004).

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Composite hydrogel:

The hydrogels investigated in this study were prepared by free-radical polymerisation using ultraviolet (UV) light. Hydrogels were synthesised using a UV curing system (Dr. Gröbel UV-Electronik GmbH). The irradiation chamber utilised was a controlled radiation source with 20 UV-tubes that provide a spectral range of between 315-400 nm at an average intensity of 10-13.5 mW/cm^2. The prepolymerised mixtures were prepared by combining desired amounts of macromolecular monomer (PEGDMA) with a specified amount of distilled water, hydroxyapatite and 0.1 wt% photoinitiator (see Table 1). The compositions of the control/composite hydrogels are listed in Table 1. The batches were placed in a 50 mL beaker, mixed using a magnetic stirrer for one hour, and finally sonicated for 30 minutes until a homogenous mixture was achieved. The solutions were pipetted into silicone moulds for further characterisation tests. Photopolymerisation was carried out for 10 minutes, after which time gelation occurred.

Table 1: Formulated composition of hydrogel composites prior to photopolymerisation

Hydrogel Code	PEGDMA 600 (wt%)	Hydroxyapatite loading (wt%)	Distilled water (wt%)	Irgacure 2959 (wt%)
A1	75	1	25	0.1
A10	75	10	25	0.1
A17.5	75	17.5	25	0.1

Fourier Transform Infrared Spectroscopy:

Fourier transform infrared spectroscopy (FTIR) spectroscopy testing of composite samples was conducted using a Perkin Elmer System 2000 FTIR microscope. FTIR was carried out using transmission mode with a 100 scan per sample cycle and a resolution of 8. The samples were scanned from 650cm^{-1} to 4000cm^{-1}. Samples for FTIR were first dried in a vacuum oven for 24 hours at 60°C to remove any moisture present.

Compression Measurement:

Compression tests were performed on a Lloyd Lr10K screw-driven testing machine fitted with a 2.5 kN load cell with a bespoke 30 mm diameter testing head. Prior to testing, hydrogels were equilibrated at room temperature for 72 hrs in buffer solution (pH 7.4). Unconfined compression tests were carried out at a speed of 0.5 mm/min and samples were strained to 60 %.

Rheological Measurements:

In all cases throughout this study, rheological measurements were performed using an Advanced Rheometer AR1000 (TA Instruments) fitted with a Peltier temperature control to investigate the comparative strength of the samples. Samples were tested in quintuplicate (using individual samples) within 72 hr of preparation at a temperature of 37°C using a 4 cm parallel steel plate where the samples were in the equilibrium swollen state. Prior to testing, all samples were blotted free of water using filter paper in an attempt to minimise slippage. A compression load of 5±0.2 N was exerted on the samples during testing and the mean ± SD was reported. Rheological test parameters, storage/elasticity (G') and loss (G'') modulus were obtained under dynamic conditions for these non-destructive oscillatory tests. Dynamic strain sweep test experiments were performed at a constant frequency of 1 Hz with percentage strain ranging from 1.80x10^{-4} to 1.0x10^{-3}.

Swelling Studies:

After photopolymerisation, the samples were placed in buffer solution (pH 7.4) to determine their water uptake after swelling. The equilibrium water content (EWC) was calculated using Equation 1:

$$\text{EWC}\ (\%) = \left(\frac{W_s - W_d}{W_d}\right) \times \frac{100}{1} \qquad \text{Eqn. 1}$$

where W_d and W_s are the weights of the hydrogels after photopolymerisation and in the swelling state, respectively. Tests were carried out in quintuplicate and data is presented as mean ± SD.

Gel Fraction Measurement:

The gel fraction of all batches was measured in quintuplicate using round discs with an average mass of 0.8 ± 0.1 g. Potassium chloride (KCl), potassium biphthalate ($C_8H_5O_4K$), potassium phosphate monobasic (KH_2PO_4), and boric acid (H_3BO_3) were used to prepare the pH 7.4 buffer solution. Hydrochloric acid and sodium hydroxide were used to adjust ionic strength of the solution to 0.2 M. The pH of the solution was confirmed using a Jenway 3520 pH meter. Hydrogels were initially dried under vacuum at 200 mmHg for 24 hr at 80°C to a consistent weight. The samples were allowed to swell in a sealed petri dish with 30 mL of buffer solution (pH 7.4) for 72 hrs at 21°C until equilibrium swelling was achieved. Once equilibrium swelling was

attained, samples were again dried in the vacuum oven at 80°C in the absence of water until no change in weight was observed. Gel fraction (%) was calculated using Equation 2:

$$\text{Gel fraction } (\%) = \left(\frac{W_{ex}}{W_o}\right) \times \frac{100}{1} \qquad \text{Eqn 2}$$

where W_o and W_{ex} are the weight of the dried hydrogel after photopolymerisation and the dried weight of the sample after extraction of soluble parts, respectively.

X-ray diffraction:

X-ray diffraction (XRD) studies on samples were carried out using a high resolution Bruker AXS D8 DISCOVER diffractometer in Bragg-Brentano geometry, with a CuK∝ monochromated beam (λ=0.15406Å) produced at 40kV and 40mA. The scanning range was from 2 to 40^o (2θ) at a step size of 0.02^o. XRD was employed to investigate the modifications induced by the incorporation of various bioceramics into the hydrogel based composites. GADDS and Eva software packages were used to analyse the recorded results.

***In Vitro Biomineralisation Study*:**

A short term *in vitro* bioactivity study was carried out in simulated body fluid (SBF) as described by Kokubo and Takadama (Kokubo and Takadama 2006). Numerous methods have previously been developed (Cho *et al*. 1995; Kokubo and Takadama 2006). However, the Kukubo method was selected based on its almost exact replication of ion concentration of human plasma. Briefly, reagent grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, and Na_2SO_4 were dissolved in 1L of distilled water at 36.5 ± 0.2 °C and pH adjusted to 7.4 with 1.0 m-HCl and Tris. Samples were immersed in SBF and kept under static conditions at 37°C for three weeks, after which they were dried at 80°C for one day and analysed by XRD to determine the deposition of apatite on the surface of the hydrogel based composites. SBF solution was changed twice a week to prevent saturation of the solution and ensure pH stability to replicate the physiological environment.

Statistical Analysis:

A statistical comparison of hydrogel composites were performed using a one way ANOVA with a Tukey's Honesty post hoc test to determine differences between individual batches. The software packages used to perform statistical analysis was SPSS version 16.

RESULTS AND DISCUSSION

Synthesis Of Hydroxyapatite:

A sample Fourier transform infrared spectroscopy (FTIR) spectrum of synthesised hydroxyapatite (HAP) is shown in Figure 1. The synthesised hydroxyapatite characteristic phosphate group peaks were observed at 1023 cm^{-1} (corresponding to PO_4^{3-} $\upsilon 3$ stretching), 962cm^{-1} (associated with PO_4^{3-}, $\upsilon 1$ stretching) and 600, 560 and 524 cm^{-1} (related to PO_4^{3-}, υ bending) (Jongwattanapisan *et al*. 2011). Synthesis of pure hydroxyapatite was confirmed by the complete reaction of constituents were confirmed by the absence of any calcium carbonate groups between 1400-1500 cm^{-1}.

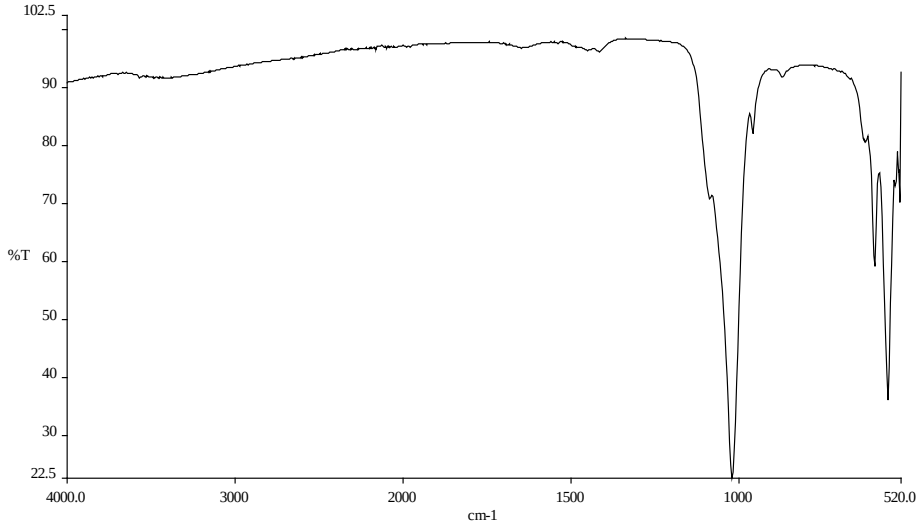

Fig. 1: FTIR spectra of hydroxyapatite

Swelling Studies:

Hydrogels are 3-D networks formed from hydrophilic polymers which are crosslinked to form insoluble polymer matrices (Slaughter *et al*. 2009). Their unique structure allows hydrogels to maintain large quantities of water. Swelling is based on the principle of occupying the free volume in a sample, until it can't swell anymore when it reaches a state of equilibrium. This is achieved by the elasticity of the polymer network and the osmotic pressure becoming equal (Neffe *et al*. 2011). Scientists must consider the swelling percentage in fabricating synthetic bone substitutes, in terms of the mechanical strength of the bone substitute material, where the decrease in swelling can positively affect the mechanical properties in a system.

In this study it was found that the percentage swelling could be controlled by altering the hydroxyapatite loading. Figure 2 shows the swelling study results for hydrogels A1-A17.5. Results show that hydrogel composite A1 had a significantly higher swelling percentage than that of hydrogel based composites A10 and A17.5 (P<0.05). This reduction in percentage swelling was due to the hydroxyapatite occupying the empty voids within the polymeric scaffold which induces greater strength in the overall hydrogel based composites.

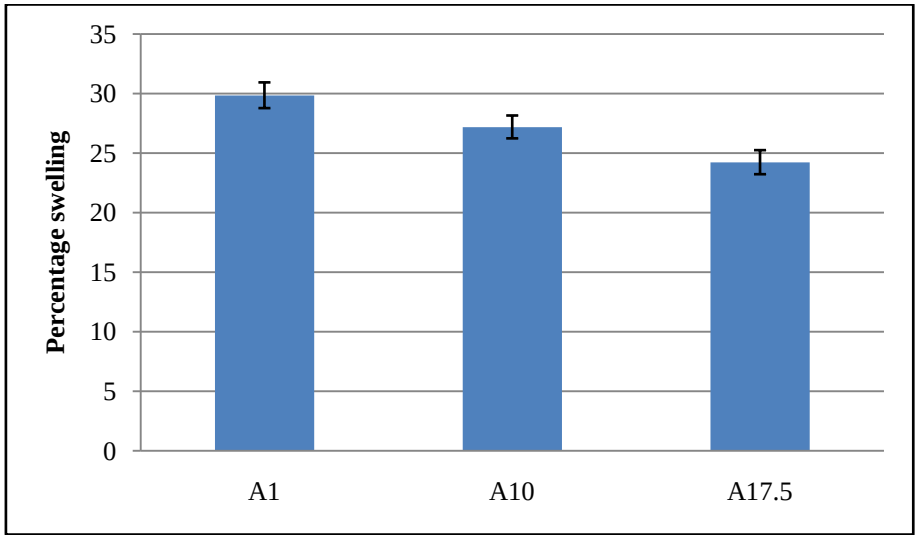

Fig. 2: Percentage swelling results for hydroxyapatite based hydrogels

Gel Fraction Measurement:

This was carried out at room temperature using circular discs. Gel fraction can be used as a quantitative indicator on the efficiency of the hydrogel network (Jing *et al*. 2001).The results for hydrogel based composites showed that increasing hydroxyapatite concentration into the precursor slightly reduces the gel fraction. For example hydrogel composite A1 had a gel fraction of 95.45 ± 0.87% compared to A10 92.28 ± 0.76% and A17.5 88.27 ± 1.25%. The reduction in gel fraction was associated with the hydroxyapatite disrupting the crosslinks between monomeric chains and thus causes the polymeric chains to have fewer opportunities to covalently link to the network. The gel fraction in general was relatively high in all hydrogels. Therefore, it can be concluded that hydroxyapatite in the prepolymerisation mixture was not able to significantly disrupt the network connectivity and in all cases gel formation occurred.

Compression Testing:

In recent years the need for alternative bone graft substitutes has develop several contenders, which include bioceramics, polymers and polymer composites. The use of bioceramics has been shown to be bioactive, osteoconductive and osteoinductive. However, the brittleness nature of these materials makes their application limited. On the other hand polymers have good capabilities in drug delivery with the incorporation of cells. However, polymers lack the compression strength and bioactive properties to that of bone. Therefore, the combination of polymer and bioceramics can promote the advantages of impact beneficial properties such as resistance to brittle failure and mechanical strength.

Results for PEG/HAP blends containing different loadings of HAP are collectively shown in Figure 3. Most of the samples exhibited a linear stress-strain region up to 30% where the compressive modulus for each sample was calculated within this linear region. Young's modulus values ranged from 7.18±0.57MPa to 8.24±0.59MPa. This increase in compressive strength was related to the decrease in the percentage swelling (see Section 3.2) and hydroxyapatite absorbing the compressive load. However, in statistical comparison between batches, the increase in strength was not significant (p>0.05).

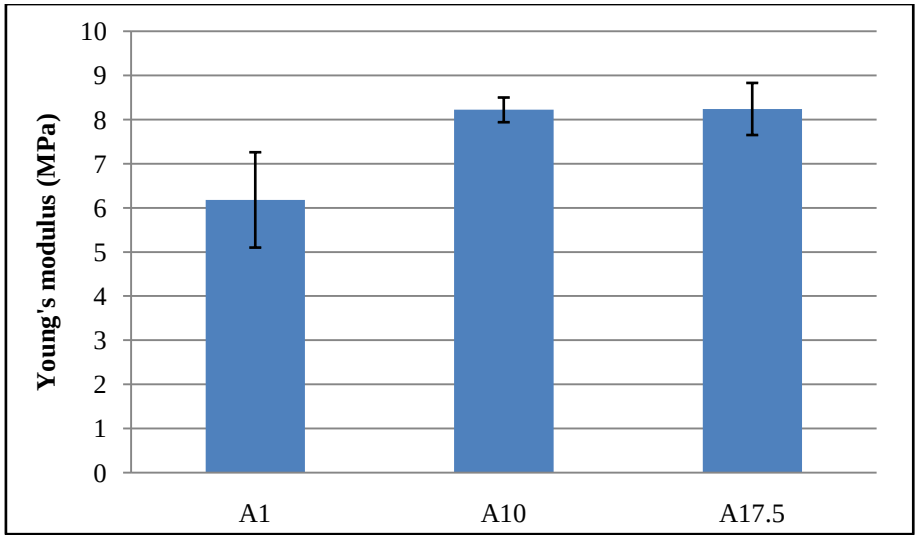

Fig. 3: Young's modulus results for hydroxyapatite based composites

Rheometry Studies:

The viscoelastic properties and mechanical strength of the hydrogels were additionally tested using an Advanced Rheometer to assess their retention behaviour and physical integrity for potential *in vivo* applications. The scaffolds viscoelastic properties were investigated based on the concept that hydrogels with good mechanical strength are expected to maintain their integrity under different loading conditions. A strain sweep test was performed on the hydrogel based composites at different loadings in order to establish the regime of linear viscoelasticity (LVE) and determine if the elasticity of the formulations differed. For all samples, G′ was over an order of magnitude higher than G″, suggesting that the hydrogel based composites were more elastic than viscous within the range tested. Storage modulus (G') values for hydrogel composites are shown in Figure 4. It is clear that the incorporation of hydroxyapatite into the polymeric system resulted in an increase in storage modulus. This increase in gel strength can be attributed to two factors. Firstly, as illustrated in Section 3.2, the incorporation of hydroxyapatite into the matrix causes a reduction in the percentage swelling for the composites. Secondly, the physical bonding present between both components causes an increase in compressive strength. In conclusion, a significant improvement in the mechanical properties was identified with the addition of hydroxyapatite.

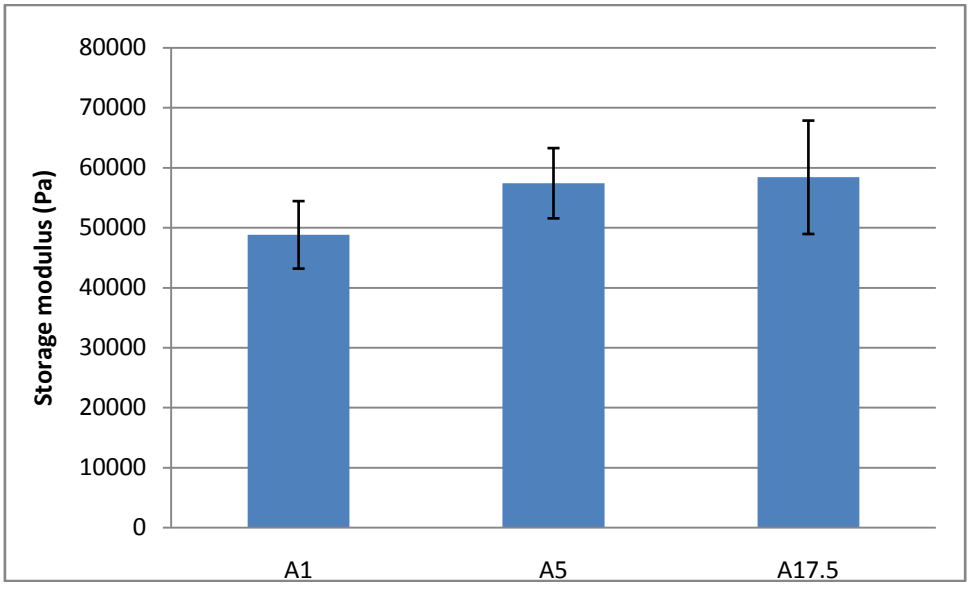

Fig. 4: Storage modulus results for hydroxyapatite based hydrogels in strain sweep mode

In Vitro Biomineralisation:

Bioactivity plays an important role in the integration of the implant with the surrounding tissue. If a synthetic material is biocompatible, the body can still reject it by encapsulating the scaffold in a fibrous tissue and thus rendering it ineffective. This process occurs unless the material has sufficient bioactive properties (Kokubo and Takadama 2006). *In vitro* biomineralisation studies can determine a material's *in vivo* osteoinductive properties by placing samples in simulated body fluid (SBF). In this study, the bioactive properties of the hydrogel based composites were studied *in vitro* by analysing the ability of the scaffold to form an apatite layer. An acellular protein free SBF, with ion concentration, pH and temperature similar to those of the human blood plasma, was employed as the medium for apatite nucleation. The formation of an apatite layer is essential in assisting a synthetic biomaterial, i.e. foreign material, to bond with the surrounding bone tissue *in vivo*. The formation of apatite was confirmed by comparing hydrogel composite A17.5 before and after immersion in SBF (Figure 5II and III), where the polymer phase at 20^{o}C almost completely disappears and new apatite peaks form at 29, 32 and 35^{o} (2θ). The formation of apatite layer takes place through a number of steps involving Ca, PO_4 and OH groups of hydroxyapatite.

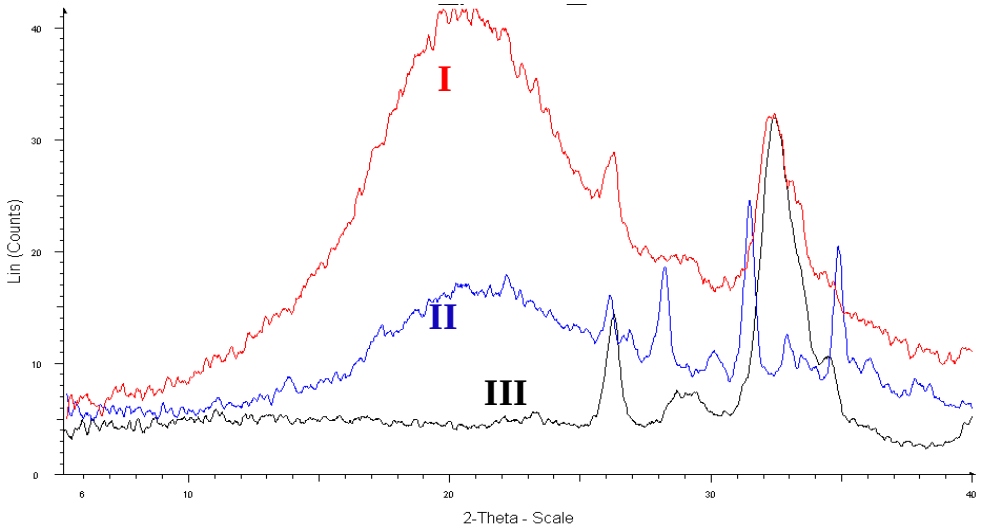

Fig. 5: X-ray diffraction of sample before (I) and after (II) immersion in SBF and HAP powder (III)

Conclusion:

Bioactivity and mechanical performance of synthetic scaffolds are of utmost importance for potential bone regeneration applications. In this study a series of polyethylene glycol and hydroxyapatite composite hydrogels were prepared via photopolymerisation. The strength of the hydrogel composites in terms of Young's modulus and storage modulus increased with the incorporation of hydroxyapatite. This increase in mechanical strength was associated with the decrease in the percentage swelling and hydroxyapatite absorbing the compressive load. The formation of apatite was confirmed through XRD where the polymer phase almost completely disappears and new apatite peaks were formed. This improvement in mechanical strength and the formation of apatite on the surface of the scaffold makes these hydrogel based composites a suitable candidate for bone graft substitutes.

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