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The Preparation Of Nanocomposite Scaffolds For Use In Bone Tissue Engineering

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Abstract: Bone is the second most frequently transplanted tissue in humans. There are 2.2 million bone graft procedures annually, which have several disadvantages such as: donor site morbidity, risk of disease transmission and a limited supply of bone. While several bone substitute materials exist, none fulfil all the requirements of an ideal bone substitute material, which should mimic as closely as possible natural bone. Therefore, the aim of this study was to develop a multifunctional nanocomposite scaffold for use in bone tissue engineering which prevents antimicrobial attachment.

Key words: Chitosan; Hydroxyapatite; Composite; Tissue Engineering.

INTRODUCTION

Biomaterials and fabrication technologies play a key role in tissue engineering. Biomaterials can be defined as any material that is used to replace or restore function to a body tissue and is continuously or intermittently in contact with body fluids (ASM 2009). Biomaterials come in synthetic and non-synthetic form. Synthetic biomaterials include certain metals, ceramics, polymers and composites (Davis 2003), while collagen and chitosan are two examples of natural or non-synthetic biomaterials. Some of the materials currently used as bone graft substitutes are not ideal due to their mechanical properties being very different from that of bone they are replacing and they also exhibit lower bioaffinity. For example, metals such as stainless steel and titanium alloys mechanically destroy the normal adjacent bones with which they are in contact, can cause allergy and inflammation due to abrasive particles and leach toxic ions such as Nickel, Cobalt, Chromium, Aluminium and Vanadium (Kikuchi *et al*. 2001). Natural ceramics however such as hydroxypaptite (HA)are biocompatible and have a similar mineral content to that bone of natural bone and teeth, (Blokhuis and Arts 2011). Indeed, HA has a long history of being used as a biomaterial in bone grafting, bone tissue engineering and bone drug delivery owing to its desirable characteristics ofbioactivity, osteoconductivity, non-toxicity, non-inflammatory response and non-immunogenicity (Murugan and Ramakrishna 2006). Natural bone, being an innate example of inorganic–organic biocomposites, consists in composition of approximately 70 wt% inorganic crystals mainly hydroxyapatite (Zhang *et al*. 2008). HA on its own however lacks properties such as mechanical strength and is brittle in composition but its advantages outweigh its disadvantages and so studies have been carried out using HA as a composite material to improve its function. For example Murugan *et al*. combined HA with the natural polymer collagen to produce a composite with an elastic modulus similar to that of bone and found that together these materials were more mechanically stable than being used as individual materials for the purpose of three-dimensional bone scaffolds.Biodegradable scaffoldsare temporary templates introduced at the defective bone site to initiate bone tissue regeneration, while it gradually degrades and is replaced by newly formed bone tissue (Thein-Han and Misra 2009). Chitosan is one such polymer which may meet these requirements and to date numerous studies have been carried out using a composite structure of HA and chitosan. In 2002, Ang *et al*. found that not only did they successfully create a 3-D scaffold but after some weeks of in vitro culturing, SEM images showed good cell attachment and proliferation(Ang *et al*. 2002). Chitosan is one of the most abundant natural polymers next to cellulose and is the *N*-deacetylated derivative of chitin found in the shells of crustaceans and insects(Demirci *et al*. 2009). Chitosan has become of great interest not only as an under-utilised resource but also as a new functional biomaterial of high potential in various fields and the recent progress in chitin chemistry is quite significant (Dutta *et al*. 2004). It has novel properties such as biocompatibility, biodegradability, antibacterial and wound-healing properties.

The aim of this current study was to prepare a composite scaffold based on chitosan and hydroxyapatite which replicates the mechanical properties of bone itself.

MATERIALS AND METHODS

Materials:

Medium molecular weight chitosan (100,000 – 300,000)were obtained from Sigma-Aldrich. Crosslinking of the scaffold was achieved through a free radical reaction of an unsaturated crosslinking agent.Calcium chloride, ammonium phosphate dibasic, ammonium hydroxide solution and chitosan were all obtained from Sigma-Aldrich. All materials were used as received.

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Hydroxyapatite Synthesis:

Hydroxyapatite was prepared using the wet chemical method with some minor changes made to the procedure(Murugan and Ramakrishna 2004). An aqueous 0.3 M ammonium phosphate dibasic $(NH_4)_2HPO_4$ solution was added drop-wise to a 0.5 M calcium chloride ($CaCl_2$) aqueous solution under continuous 1000rpm stirring at 60^0C for one hour. The pH was maintained above pH 10 by adding ammonium hydroxide solution drop wise. The heat was then turned off and the solution continued stirring for another 30 minutes. The mixture was left to allow the HA to precipitate out of solution overnight. The solution was then decanted and the precipitate was filtered and washed several times with distilled water to rid of any residual NH_4OH. The white paste was then microwave irradiated for 10 minutes using a domestic microwave oven, (Sharp – R-33ST, 900W). The resultant material was then placed in a vacuum oven at 50mbar for 24 hours at 60^0C to dry. After 24 hours, the material was ground to a fine powder using a pestle and mortar before sieving through a micro filter with a pore size of 50 microns to produce micro sized HA particles.

Scaffoldsynthesis:

Chitosan (CS)and hydroxyapatite powder (HA) of known quantities as outlined in Table 1 were dry mixed and added to this was a known amount of acidic solution of acetic acid and distilled water. The composites scaffolds were subsequently crosslinked. The cured samples were covered with parafilm and stored overnight at room temperature before characterising and testing the samples.A total of five different batchesas defined using Design of Experiments (DOE) approach were used for this study, each batch contained different ratios of the base materials, Table 1. Each batch made was divided into circular moulds and each sample weighed approximately 1.2mg. Batch number three was synthesised and half the batch subjected to free radical-photocrosslinking reaction and the second half was not subject to this as it was used for testing which will be discussed later in the paper.

Table 1: Batches synthesised and analysed for this test and their base material ratios

Batch No.	Chitosan:HA	Crosslinking agent	Initiator solution
		(g)	(g)
1	1:2	0.2	1
2	1:1	0.2	1
3	1:2	0.02	1
4	1:1	0.02	1
5	1:1	0.2	0.1

*crosslinking agent and photoinitiator solution are as a percentage of chitosan

Characterisation Methods:

Drying Study:

Samples were placed into petri dishes in a vacuum oven, (Salvis Lab, 1000mbar) at 50mbar and left to dry at 30^0C. This temperature was selected as preliminary studies carried out found this temperature to be the most effective without causing degradation of the samples. The samples were removed from their petri dishes and weighed at the following time points:

0hrs, 1hrs, 2hrs, 4hrs, 6hrs, 24hrs, 26hrs and 28hrs. From preliminary tests, it was noted that after approx. 24 hours samples began to degrade as observed through discolouration of the samples.Hence the samples were removed from the oven after the 24 hour time point at which time their weights had reached equilibrium. From the recorded sample weights, the percentage weight loss of each sample was calculated using the formula:-

$\%\ \text{Weight loss} = ((W_0 - W_t)/W_0) \times 100$

Where W_t is the weight of the sample at a predetermined time and W_0 is the weight of the sample before drying studies took place.

Swelling Studies:

Three samples from each batch which had been dried to equilibrium were placed in petri dishes which were filled with Phosphate Buffer Solution (PBS), pH 7.2 +/- 0.2 at 37^0C, to replicate *in vivo* swelling conditions. Periodically the samples were removed from the PBS; pat dried using filter paper and their weights recorded. The samples were then re-submerged in buffered solution and left to swell until the samples had reached equilibrium. The buffer was topped up as required throughout the swelling study to ensure the samples were constantly immersed in the solution. Both the water content (W_c) and the water uptake (W_u) of the composites were also calculated using the following formulae:

$$W_c = \frac{100(W_t - W_0)}{W_t}$$

&

$$W_u = \frac{100(W_t - W_0)}{W_0}$$

Compression Testing:

Compression testing was performed on a Lloyd Lr10K, screw driven testing machine fitted with a 2.5kN load cell with a bespoke 30mm diameter testing head. Compression tests were performed in order to calculate Young's modulus and stress at limit parameters of the samples for comparison to those of natural bone. Testing was carried out using 6 samples in each batch. All samples were compressed to 60% of their original height at a compression rate of 0.5mm/min.

Contact Angle Testing:

Contact angle measurements were performed using a goniometer, (FTA 1000 Analyzer Systems) to assess the relative hydrophobicity and/or hydrophilicity of selected samples. In this test a 1mL droplet of water was placed onto the surface of the scaffold (sessile drop method), a camera then captured ten separate images of the droplet over a 15 second time period. The contact angle θ (degrees) is measured as the tangent drawn to the surface of the droplet. The value indicated is an average angle for all ten measurements (or smaller number if an error occurred during measurement) for both the left and right extremities of the droplet. Tests were carried out on all fivebatches of samplesin duplicate with three separate droplets of water analysed on each sample type.

Differential Scanning Calorimetry:

Differential scanning calorimetry (DSC) was carried out using a TA instruments 2010 DSC. Samples of between 8 and 12 mg were weighed out using a Sartorius scale having a resolution of 10^{-5}g. All measurements were conducted in non-sealed non-hermetic aluminium pans to allow for any trace water in the sample to escape from the pan. The samples were heated from 20 to 300^0C at a heating rate of 10^0C/min, under a 30mL per minute flow of nitrogen to prevent oxidation. Analysis was conducted on five batch types in triplicate and the glass transition temperature (Tg) were recorded for each batch if present.

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was performed on a Mira SEM with a magnification of 10kx using a Tescan/MIRA back scattered detector. The samples tested were sectioned into thin slices with a razor blade. The samples were placed on an aluminium stub and were gold coated using Baltec SCD 005 for 110 sec at 0.1 mBar vacuum to give a final gold coating thickness within a working range of 10-30 nm prior to testing. The purpose of performing SEM testing was to analyse how well dispersed the HA was throughout the composite sample and also to determine the dimensions of the HA particles.

To determine the elemental composite of the HA particles, an Energy-Dispersive X-ray spectrometry (EDX) detector was employed.

RESULTS AND DISCUSSION

Drying Study:

All samples retained their shape throughout the drying test however the overall size of the samples did reduce due to water loss. It was noted that the 24 hour time point was the most suitable time to remove the samples from the oven for further testing as after this point was reached the samples began to degrade. Samples containing a lower concentration of chitosan and a higher concentration of HA, crosslinking agent and photoinitiator solution lost the least amount of weight with the lowest percentage water loss values (Table 2). This was most likely due to the HA filled chitosan pores preventing the chitosan matrix from contracting. It was observed that the samples which lost the most weight were those that contained a higher concentration of chitosan and a lower concentration of HA, crosslinking agent and photoinitiator solution (Figure 1). This was most likely due to less HA present within the pores of the chitosan which lead to contracting of the chitosan matrix in the sample. Samples with an equal ratio of chitosan and HA also resulted in a higher percentage water lossvalue than those with a higher HA ratio.

Table 2: Drying study - percentage water loss of samples

Sample no.& CS/HA ratio	Weight (g) at 0 hrs	Weight (g) at 24 hrs	Water loss (%)
1 (1:2)	2.68	1.46	45.5
2 (1:1)	2.93	1.37	53.2
3 (1:2)	2.87	1.54	46.3
4 (1:1)	2.54	1.23	51.6
5 (1:1)	2.53	1.27	49.8

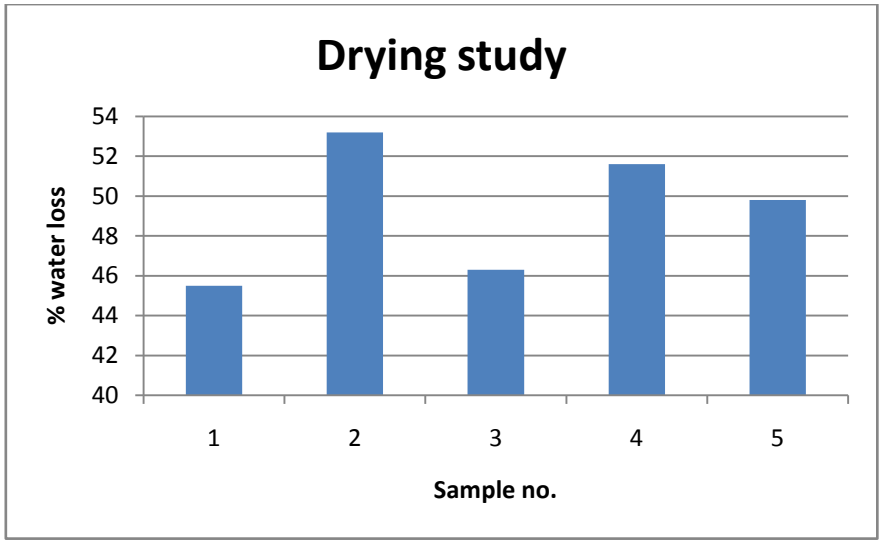

Fig. 1: Drying study - percentage water loss of samples

Swelling Study:

All samples were swelled for a total of 528 hours (22 days). At the 24 hour mark all five batches had swelled to their saturation point. Samples containing a higher concentration of hydroxyapatite resulted in both lower water uptake and water content values compared to those which contained lower HA concentrations, (Table 3). Composites containing equal amounts of chitosan and HA (Table 1) swelled consistently higher than those containing a higher concentration of HA (Figure 2).

Table 3: Swelling study - percentage water uptake and water content of samples

Batch no. & CS/HA ratio	Weight (g) 0 hrs	Max. Swollen weight (g) – 24hours	Water Uptake - W_u (%)	Water Content - W_c (%)
1 (1:2)	1.34	4.86	262.6	72.4
2 (1:1)	1.29	8.40	551.2	84.6
3 (1:2)	1.43	7.51	425.2	81.0
4 (1:1)	1.13	6.04	434.5	81.3
5 (1:1)	1.20	5.92	488.3	79.7

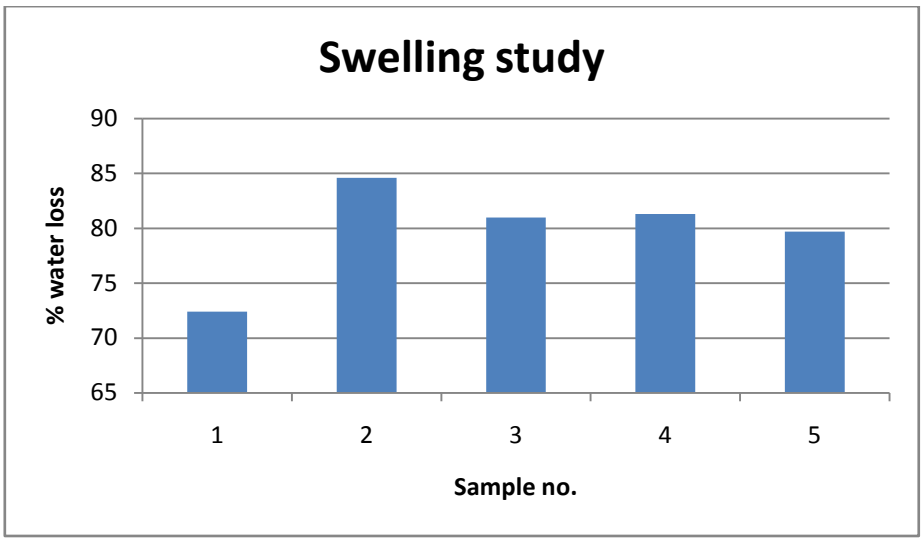

Fig. 2: Swelling study - percentage water loss of samples

Samples containing a ratio of 1:2 (CS/HA) swelled to between 171% and 415% during this study and held their shape throughout. Their swelling is likely to have been retarded as they contained a higher ratio of HA which was dispersed between the chitosan polymer chains. Therefore less PBS was able to penetrate the samples within the crosslinked chitosan material as these pores were filled with HA, hence preventing them to swell further. Samples with a ratio of 1:1 (CS/HA) swelled to 655% compared to 1:2 (CS/HA) samples which swelled to just 415% of their original dry weight, (Figure 3).

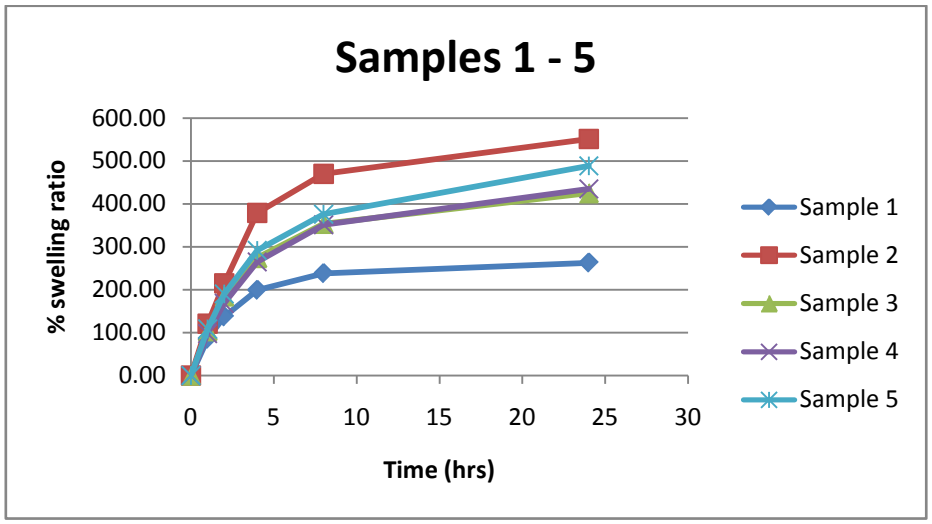

Fig. 3 Swelling ratio of samples 1 - 5

Samples 1:1 (CS/HA) contain less HA than samples 1:2 (CS/HA), hence they were more porous and retained more buffered solution within these pores. However, these samples appeared brittle and soft to touch therefore indicating their strength had been compromised after swelling. It was noted that samples containing a higher concentration of chitosan and a lower concentration of HA, crosslinking agent and photoinitiator solution swelled more than those with a lower chitosan concentration and a higher concentration of HA, crosslinking agent and photoinitiator solution (Table 1). The latter samples when handled also had a more robust structure and shape retention.

Compression Test:

Batches 1-5 were compression tested to determine their Young's modulus values and stress at limit values.The mechanical properties of the samples were quantified using compression testing following 10 days in PBS, testing was carried out on six samples per batch and their averagevalues obtained,(Figure 4).

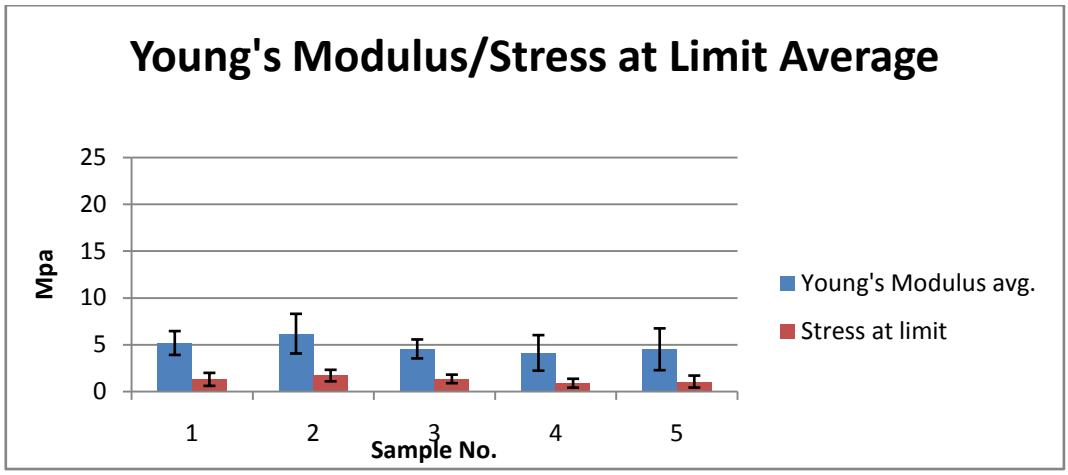

Fig. 4 Young's modulus and stress at limit average values for batches 1 - 5

Batches three, four and five yielded results below the mechanical properties of natural cancellous bone which has a Young's modulus value of 50–500MPa and a stress at limit value in the range of 7 – 10MPa (Murugan and Ramakrishna 2005). Batches one and two yielded results closest to those of cancellous bone with average Young's modulus values of 5.17MPa and 6.17MPa and stress at limit values of 1.29MPa and 1.69MPa. These results are an improvement on the 4.12 – 4.54MPa Young's modulus values and the 0.88MPa – 1.34MPa stress at limit values that were recorded for samples three to four. Samples one and two contained a higher concentration of both crosslinking agent and also photoinitiator which resulted in a higher crosslinking density within the samples and therefore a more mechanically stronger result was achieved. The higher crosslink density would prevent PBS infiltration thus reducing the percentage swelling as shown with sample 1. However as

sample 2 had the highest percentage swelling, its mechanical properties can be attributed to uneven mixing of the base materials in this batch prior to the samples being divided into the moulds and subjected to free-radical photocrosslinking.

Contact Angle Test:

The wettability of a surface is usually expressed in terms of contact angle(Jae Hyun Park 2009). This is the angle at which the liquid - vapour intersurface meets the solid surface. Depending on the surface, the surface can be classified as been superhydrophilic to superhydrophobic (Figure 5).

$$\begin{cases} \theta_\infty > 150^\circ & \text{superhydrophobic,} \\ 65^\circ < \theta_\infty < 150^\circ & \text{hydrophobic,} \\ 0^\circ < \theta_\infty < 65^\circ & \text{hydrophilic,} \\ \theta_\infty \approx 0^\circ & \text{superhydrophilic,} \end{cases}$$

Fig. 5: Criteria for determining the relative hydrophilicity/hydrophobicity of a surface

Where the subscript ∞ refers to the contact angle of a droplet whose size is sufficiently large, (Jae Hyun Park 2009).

In the current study the contact angle for the samples tested ranged from 44 to 88 degrees which shows a broad range of hydrophobicity and hydrophilicity, (Figure 6). The exact surface characteristics required for optimal osteointegration remain to be elucidated (Kieswetter et. Al, 1996). However, none of the samples tested in this study were in the superhydrophilic or superhydrophobic regions, which are not be suitable for cell attachment once the sample is implanted. It has been noted from the literature that cells such as osteoblasts become activated when in contact with rough surfaces as compared with smooth and polished surfaces (Beatrice Sommer 2004). In 2010,J.S. Hayesshowed that bone cell attachment could be regulated through surface modifications of the surface roughness of Titanium (Ti) implants. Hayes et. al showed that osteogenic cells as well as other bone forming cells migrate via fibrin to the fracture site during the fracture healing process. It is via the attachment of this fibrin to the implant surface that the cell attachment process begins, however the author found that fibrin attached more readily to micro rough surfaces than smooth surface implants Therefore, the composite scaffolds developed in this work should not only be suitable for cell adhesion but also their biocompatibility is also an added advantage for their possible use for bone tissue engineering.

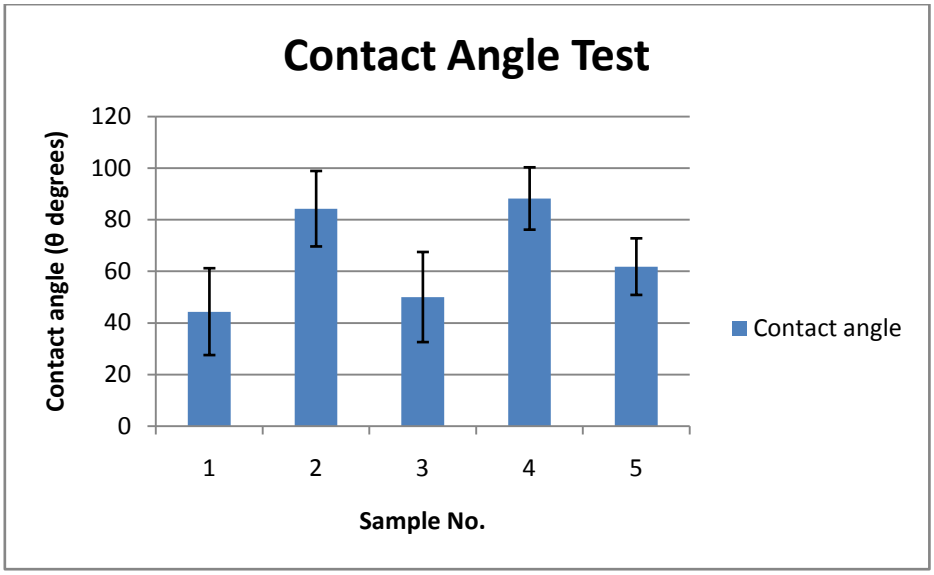

Fig. 6: Contact angle test results - average of all samples. Results indicate mean with error bars indicating standard deviation

Differential Scanning Calorimetry:

From a review of the literature there are conflicting reports on the Tg value for chitosan. The reported values are 150^{0}C, 161^{0}C and 203^{0}C, (Yanming Dong 2004). DSC analysis was carried out on all five samples in triplicate and the resulting thermograms for each sample were overlaid on one graph for each separate batch. Thermograms of batches 1 - 5 depicted no evidence of a Tg and all samples resulted in consistent thermograms,

(Figure 7). From analysis of these thermograms, no transition was detected at any of these temperatures. The large peaks present at approx. 100^0C are associated with the water phase of each sample evaporating. According to (Monmaturapoj 2008), the Tg of HA ranges from 430^0C - 480^0C, hence the shift in any of the peaks of the batches tested are too low to be in relation to the Tg of chitosan. The glass transition temperature is a measure of the mobility of the side chains of a compound and it is therefore most likely that none of the above batches tested have a Tg as their chains have been crosslinked and are not mobile. These test results help to confirm that crosslinking between chitosan chains has taken place.

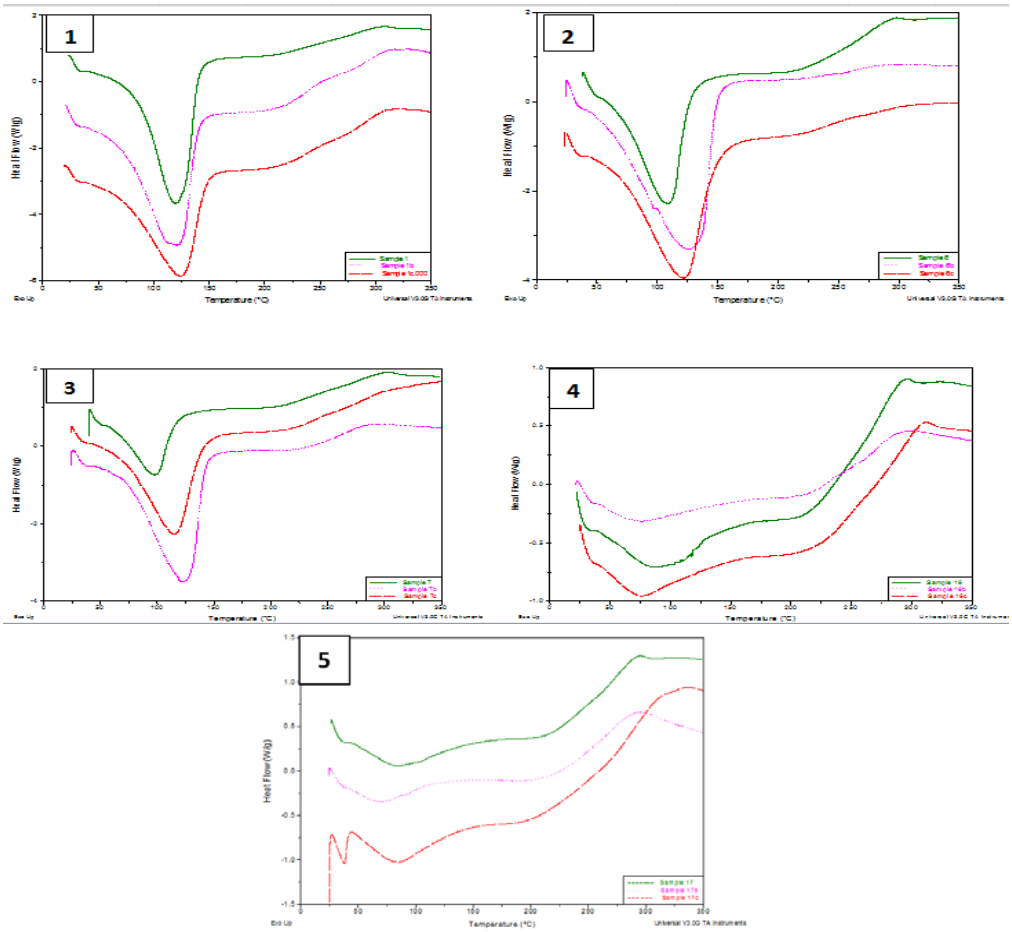

Fig. 7: DSC thermograms - batches 1 - 5 tested in triplicate

Scanning Electron Microscopy:

Sample one which was analysed under an SEM to assess the dispersion of HA within the chitosan matrix. This test revealed good dispersion of HA throughout the sample. SEM was also used to assess the approximate dimension of some HA particles, Figure 8. While some hydroxyapatite particles can be seen as individuals, the majority of the HA material appears to be bound together within the sample. Chitosan was also clearly visible in the image. CS appeared to be dispersed throughout the image hence further confirming that both materials, CS and HA were quite well distributed throughout the sample.

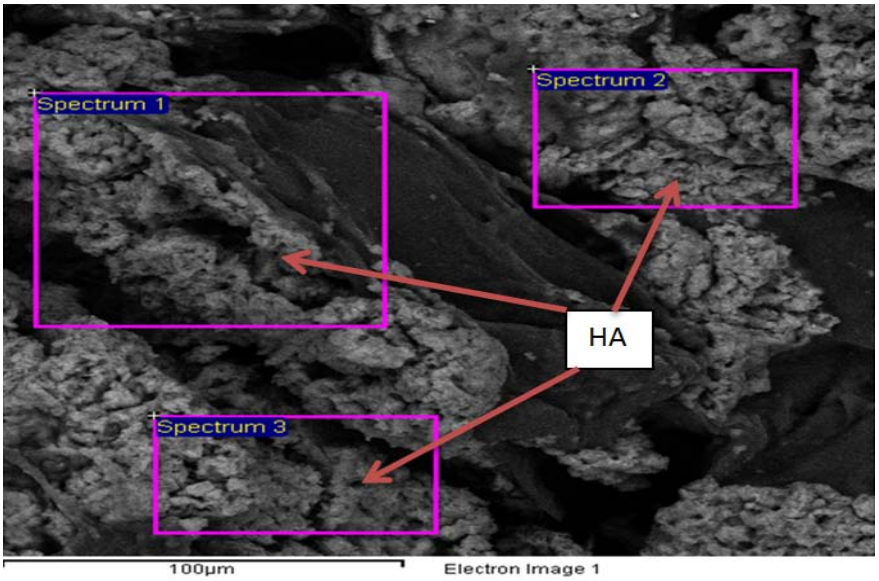

Fig. 8: SEM image of sample 1 (1:2 CS/HA)

Sample one was also analysed under EDX to determine the elemental composition within the composite. Elements Carbon (C), Oxygen (O), Phosphate (P) and Calcium (Ca) were all present within the sample, Figure 9. According to (Murugan and Ramakrishna 2004) both calcium and phosphorus are primary elements of HA while the presence of carbon and oxygen may be attained from the chitosan molecules. Trace elements of Sodium (N) and Potassium (K) were also visible on the spectrum, however this was most likely due to the samples previous immersion in PBS buffer.HA made from ammonium phosphate dibasic and calcium chloride, showed the absence of chlorine (Cl) and nitrate (N) indicating a high level of purity in the sample.

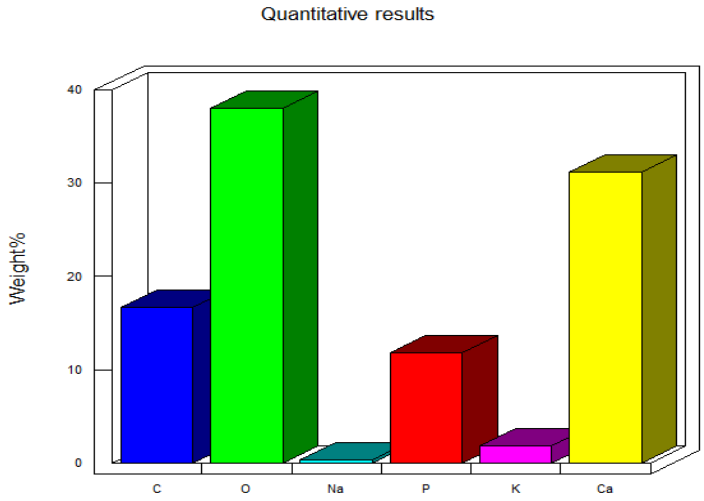

Fig. 9: Elemental composition of sample 1

Conclusion:

In total, five different composite scaffolds were synthesised. It was evident that samples with a higher ratio of HA, crosslinking agent and photoinitiator solution were the most promising samples for use as a bone substitute material. These samples had a lower percentage swelling which imparted greater mechanical strength and their shape, handle ability and composition was not greatly affected by swelling. The sample containing a higher ratio of chitosan to HA and a lower concentration of crosslinking agent and photoinitiator solution swelled to a higher degree most likely due to a number of factors;

1. Less crosslinking agent and photoinitiator solution resulted in less crosslinking, hence larger pores in the chitosan matrix
2. Lower amount of HA allowed for more free space between crosslinked chains of chitosan which were then replaced with the buffer solution

Although their compressive strength was less than natural bone, this can be improved with possibly the addition of more HA and/or a higher concentration of crosslinking agent and photoinitiator solution. Aside from

compressive strength, hydrogels show other promising characteristics such as their drug loading and dissolution possibilities due to their porous structure. They are less brittle than ceramics, they are easy to process and their flexible structure allows for a higher stress tolerance, something metallic materials lack, (Cheung *et al*. 2007).

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