Research Paper

Incorporation of the natural marine multi-mineral dietary supplement Aquamin enhances osteogenesis and improves the mechanical properties of a collagen-based bone graft substitute

Orlaith Brennan[a,b,c,*], Barry Stenson[a,b], Amro Widaa[a,c], Denise M. O'Gorman[d], Fergal J. O'Brien[a,b,c]

[a]Tissue Engineering Research Group (TERG), Royal College of Surgeons in Ireland, Dublin 2, Ireland
[b]Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland
[c]Advanced Materials and BioEngineering Research Centre (AMBER), Trinity College Dublin and Royal College of Surgeons in Ireland, Dublin 2, Ireland
[d]Marigot Ltd., Strand Farm, Currabinny, Carrigaline, Co. Cork, Ireland

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ABSTRACT

Aquamin is a commercially-available supplement derived from the algae species Lithothamnion, which has proven osteogenic potential. By harnessing this potential and combining Aquamin with a collagen scaffold, with architecture and composition optimised for bone repair, the aim of this study was to develop a natural osteo-stimulative bone graft substitute. A fabrication process was developed to incorporate Aquamin into scaffolds to produce collagen–Aquamin (CollAqua) scaffolds at two different Aquamin concentrations, 100 F or 500 F (equivalent weight% of collagen or five times the weight of collagen respectively). CollAqua constructs had improved mechanical properties which were achieved without reducing the scaffold's permeability or porosity below the minimum level required for successful bone tissue engineering. The fabrication process produced a homogenous Aquamin distribution throughout the scaffold. Release kinetics revealed that in the first 12 h, the entire Aquamin content was released from the 100 F however, less than half of Aquamin in the 500 F was released with the remainder released approximately 21 days later giving an initial burst release followed by a delayed release. Osteoblasts cultured on the CollAqua scaffolds showed improved osteogenesis as measured by alkaline phosphatase, osteopontin and osteocalcin expression. This was confirmed by increased mineralisation as determined by von Kossa and Alizarin red staining.

In conclusion, a cell and growth factor free collagen-based bone graft substitute with enhanced mechanical properties has been developed. The addition of Aquamin to the

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*Correspondence to: Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland. Tel.: +353 1 402 2375; fax: +353 1 4022355.
E-mail address: obrennan1@rcsi.ie (O. Brennan).

collagen biomaterial significantly improved mineralisation by osteoblasts and results in a new product which may be capable of enhancing osteogenesis to facilitate bone repair in vivo.

1. Introduction

Disease, developmental disorders, tumour resection, injury and trauma can all lead to loss, degeneration and damage of bone tissue. In many cases of injury, restoration of alignment and fixation is sufficient to induce healing and repair of the tissue (Finkemeier, 2002). However, in some cases the defect or damage, whether pathological or trauma-induced, can be too extensive leading to inadequate healing. In cases such as these, bone grafting is required to provide mechanical support, fill osseous voids and to induce and facilitate bone repair (McAuliffe, 2003). Over 2.2 million bone-grafting procedures are undertaken worldwide annually to repair defects in orthopaedics, dental surgery and neurosurgery at a great socio-economic cost (Giannoudis et al., 2005). This makes bone grafts second only to blood transfusions on the list of transplanted material globally. Collagen is a major component of the bone extra cellular matrix and is inherently biocompatible and biodegradable. One major problem with using collagen as the main constituent of a scaffold for orthopaedic tissue engineering is that it has relatively poor mechanical properties (O'Brien, 2011). However, the Tissue Engineering Research Group in RCSI have demonstrated that the compressive and tensile mechanical properties of collagen and collagen glycosaminoglycan (collagen GAG) scaffolds, can be improved through physical and chemical cross-linking methods (Haugh et al., 2011; Tierney et al., 2009). In addition, we have demonstrated the optimal composition for osteogenesis and optimal pore structure to facilitate bone tissue formation (Tierney et al., 2009; Byrne et al., 2008; Murphy et al., 2010; Murphy and O'Brien, 2010; O'Brien et al., 2007, 2005). Taken together, these studies have led to the development of a collagen GAG scaffold with an optimised composition, cross-linking density and pore size for bone regeneration and we have demonstrated the ability of these scaffolds to heal bone defects in vivo in minimally loaded calvarial defects (Alhag et al., 2011; Lyons et al., 2010).

Aquamin is a commercially-available food supplement which is derived from the calcified skeletal remains of the red marine algae species Lithothamnion found off the coast of Ireland and Iceland. It is available commercially as Aqaumin F, S or soluble with calcium contents of approximately 31%, 17.5% and 12% respectively as well as containing 74 other trace minerals such as strontium, manganese, selenium, copper and zinc. Minerals from seawater accumulate in the fronds of the algae during its lifetime until eventually the fronds break off and the calcified remains are harvested and processed. The supplement does not contain any additives; the mineralised fronds are simply sterilised, dried and milled to produce the Aquamin supplements.

While autologous bone grafting or a synthetic biomaterial are viable options for bone formation, often an additional stimulus is needed to enhance osteoinduction. Recent trials have shown that Aquamin has several positive effects on bone cells and bone health when taken as a supplement and when used directly on cells. Osteoblast cells cultured in the presence of Aquamin exhibited early osteogenic potential and produced more mineral (O'Gorman et al., 2012; Widaa et al., 2014).

Dietary supplementation with Aquamin has been shown to preserve bone structure and function in mice on a high-fat Western diet (HFWD) (Aslam et al., 2010). Indeed the bone structure of mice on a HFWD with Aquamin supplementation was superior to that of mice on the standard low fat diet. Thus, indicating that Aquamin supplementation has positive effects on bone health. In a study conducted in yearling horses, bone turnover was increased with dietary supplementation with Aquamin which suggests that old or damaged bone could be replaced or removed which could, in turn, reduce incidents of clinical bone injury (Nielsen et al., 2010). Aquamin has also been shown both to increase the range of movement and walking distance and decrease the symptoms of knee osteoarthritis (Frestedt et al., 2009, 2008). All of these results suggest that Aquamin has the potential to act as a supplement to enhance bone formation.

By harnessing the osteogenic potential of Aquamin and combining it with a collagen–GAG scaffold with an architecture and composition optimised for bone repair, this study aimed to develop a natural osteo-stimulative bone graft substitute. The specific aims of this study were to develop a fabrication technique to incorporate Aquamin into a collagen scaffold and to investigate whether the incorporation of Aquamin into a collagen scaffold could produce a cell and growth factor free bone graft substitute with improved mechanical properties that could support and promote the proliferation of osteoblasts.

2. Materials and methods

2.1. Scaffold fabrication

Two scaffold groups were used in the study: collagen glycosaminoglycan scaffolds and collagen–Aquamin scaffolds (CollAqua). Scaffolds were produced by lyophilisation of a collagen GAG suspension based on a previously developed protocol (Haugh et al., 2011). Briefly, a suspension was produced by blending microfibrillar bovine tendon-derived collagen (0.5 wt%) (Integra LifeSciences, Plainsboro, NJ) with chondroitin-6-sulphate (0.05 wt%), isolated from shark cartilage (Sigma-Aldrich, Dublin, Ireland) in 2 M acetic acid. CollAqua scaffolds were fabricated by the inclusion of Aquamin during the blending process. The three Aquamin variants with varying calcium concentrations (soluble, F and S) were trialled. In order to produce high quality scaffolds, a number

of changes were made to the protocol including adjusting acetic acid molarity, the addition rates and order of addition. Despite these modifications, it was not possible to produce good quality scaffolds using Aquamin soluble and S and these variants were not continued. However, Aquamin F (highest calcium content) was successful and was added at either the equivalent weight% of collagen (100 F, 100 wt%) or five times the weight of collagen (500 F, 500 wt%).

Suspensions were maintained at 4 °C during blending to prevent denaturation of the collagen. The suspension was lyophilised using a freezing process that was described previously (Haugh et al., 2011). A final temperature of freezing of −10 °C was used in order to produce homogeneous scaffolds with a mean pore size of 325 µm (Murphy et al., 2010). After freeze-drying, the scaffolds were cross-linked using a dehydrothermal treatment by placing the samples in an aluminium foil packet inside a vacuum oven (Vacucell 22; MMM) under a vacuum of 0.05 bar at a cross-linking temperature of 105 °C.

2.2. Material characteristics

2.2.1. Effect of Aquamin on mechanical properties
Compressive testing was used to determine the effect of Aquamin F on the modulus of the scaffolds. Mechanical testing of scaffold samples was carried out using a mechanical testing machine (Z050; Zwick/Roell) fitted with a 5 N load cell. Samples were tested in a bath of phosphate buffered saline. For unconfined compression testing with impermeable, un-lubricated platens, samples of 8 mm diameter were cut from the scaffolds using a punch. Testing was conducted at a strain rate of 10%/min. The modulus was defined as the slope of a linear fit to the stress–strain curve over 2–5% strain.

2.2.2. Aquamin retention in the scaffold
A calcium assay was used to investigate the release kinetics of Aquamin diffusion from the CollAqua scaffolds in an aqueous environment. Due to the high calcium concentration in the Aquamin F (>30%) calcium release was used as an indicator of Aquamin release. 12.5 mm diameter scaffolds were immersed in 5 ml of distilled water and the scaffolds were removed after 1, 5, 15, 30, 60 and 120 min and latter time points of 12, 24, 48 and 72 h. 10 µl of each sample was added in duplicate to two wells in a 96-well plate (Sarstedt, Germany) with 100 µl Total Calcium base reagent (Stanbio, USA) and 100 µl Total Calcium colour reagent (Stanbio, USA). The absorbance was read at 570 nm using a Varioskan Flash spectrophotometer (Thermo Scientific, USA).

2.2.3. Aquamin distribution in the scaffold
Microcomputed tomography (µCT) using a µCT-40 scanner (Scanco, Switzerland) and associated software was performed to obtain 3D reconstructions of scaffolds to visualise the Aquamin distribution within the scaffolds. Samples were divided into 209 slices and high resolution imaging (8 µm) was used. Each scaffold was scanned three times and the images averaged to reduce interference. The highest energy (70 kVp) and current (114 µA) settings were used.

2.2.4. Effect of Aquamin on scaffold porosity
The porosity of each scaffold was determined using Eq. (1) below. The scaffold diameter and height were measured using Vernier callipers and the mass was measured, from which the scaffold volume and density were determined.

$$100 - \left(\frac{\rho_{Sample}}{\rho_{Material}} \right) \times 100 \qquad (1)$$

where ρ_{sample} is the density of the sample and $\rho_{material}$ is the density of the collagen (collagen GAG samples) or the collagen–GAG–Aquamin composite. The value used for the bulk density of collagen was 1.343 mg/mm^3 and for Aquamin F was 0.8 mg/mm^3. The density for the collagen–Aquamin composite was calculated using Eq. (2).

$$\frac{m_{Col} + m_{Aq}}{((m_{Col}/\rho_{Col}) + (m_{Aq}/\rho_{Aq}))} \qquad (2)$$

High permeability is important as it facilitates improved cellular infiltration and vascularisation which enhances the osteogenic potential of the host cells resulting in increased bone formation.

2.2.5. Effect of Aquamin on scaffold permeability
A specialised rig designed in RCSI was used to calculate the scaffold permeability. The rig measures the rate at which liquid passes through the scaffold and using Eq. (3) the permeability was calculated:

$$k = \frac{Q\,h\,\mu}{\Delta P\,A} \qquad (3)$$

where k is the Darcian permeability [m^4/Ns], Q is the volumetric flow rate [m^3/s], h is the height or thickness of the scaffold [m] measured before testing using digital Vernier callipers, μ is the viscosity of water [Pa s], ΔP is the pressure head applied by the water column [Pa] and A is the cross-sectional area of the scaffold [m^2] through which water flows, measured using digital Vernier callipers on removal of the scaffold from the rig after the test.

2.3. Biological characteristics

2.3.1. Effect of Aquamin on cell attachment
A cell attachment study was conducted using the pre-osteoblast MC3T3-E1 cell line. Collagen GAG, 100 F and 500 F scaffolds were placed in 6-well plates, one scaffold per well and 200 µl of 5×10^5 cell/ml MC3T3 suspension was seeded on each side of the scaffold with a 30 min interval between each side. The seeded scaffolds were incubated for a further 30 min and each well was then flooded with 5 ml of culture media containing α-MEM, 10% foetal bovine serum (FBS) and 2% penicillin–streptomycin. Scaffolds were incubated for 1, 3 or 7 days at 37 °C and 5% CO$_2$ with a change of media after 3 days. After incubation, scaffolds were washed in PBS before digesting in a solution prepared from papain enzyme solution containing 0.5 M EDTA, cysteine-HCL and 1 mg/ml papain enzyme (Carica papaya, Sigma-Aldrich, Arklow, Ireland). Cell number was quantified using a Hoechst dye 33 258 assay which fluorescently labels double stranded DNA as previously described (Murphy et al., 2010). Measurements were taken from a fluorometric plate reader (Wallac 1420 Victor2 D, PerkinElmer, MA, USA) at an emission of 460 nm and

excitation of 355 nm, 1.0 s. The measurements were read against a standard curve to obtain the relative cell numbers per scaffold in terms of the DNA content.

2.3.2. Effect of Aquamin on osteogenic potential and mineralisation

Collagen GAG, 100 F and 500 F scaffolds were each seeded as above and incubated for 1, 7, 14, 21 or 28 days. Cells were cultured in culture media supplemented with 100 nM dexamethasone, 50 µM ascorbic acid and 10 mM β-glycerophosphate to encourage mineralisation. Cell-free scaffolds were included as controls for each group.

2.3.3. Effect of Aquamin on alkaline phosphatase protein expression

For alkaline phosphatase (ALP) staining, scaffolds were flash frozen in liquid nitrogen before a lysis buffer (0.1 M Sodium Acetate Buffer and 2% Triton-x-100) was added. The scaffold was homogenised in the buffer and left on ice before being centrifuged at 4 °C for 10 min. The supernatant was removed and plated with 10 mM P-nitrophenyl phosphate. These were left in the dark for 1 h at 37 °C before the reaction was stopped with sodium hydroxide solution. Measurements were taken from a fluorometric plate reader (Wallac 1420 Victor2 D, PerkinElmer, MA, USA) at 405 nm absorbance.

2.3.4. Effect of Aquamin on osteopontin and osteocalcin protein expression

To stain for osteopontin (OPN) and osteocalcin (OCN), 10-µm sections of scaffold were deparaffinised as before. Slides were washed for 10 min × 3 in washing solution (PBS, 0.1% tween) (Sigma-Aldrich), before applying a blocking solution (PBS, 0.1% tween, 5% FBS) (Sigma-Aldrich) to the sections for 2 h. Primary rabbit polyclonal IgG antibodies against OPN and OCN (Santa Cruz Biotechnology, USA) were applied in blocking buffer at a dilution of 1:250, before leaving overnight at 4 °C. The next day, slides were washed as previously described before applying a species specific goat anti-rabbit IgG secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, USA) to the sections in the blocking buffer at a dilution of 1:250 and left in the dark at room temperature for 2 h. Slides were then washed as previously described before mounting a cover slip using VectashieldR (Vector Laboratories Ltd., United Kingdom), which stains nuclei of cells with fluorescent Dapi. Images were taken using a camera (Nikon Eclipse 90i) and a digital imaging system (NIS Elements BR 3.0, Nikon).

2.3.5. Effect of Aquamin on mineralisation

After incubation, tissue-engineered constructs were fixed in 4% paraformaldehyde for 30 min. Dehydration and paraffin embedding was carried out using an automated tissue processor (ASP300, Leica) and blocks were cut into 10-µm sections (RM2255, Leica). All stained sections were taken in a horizontal plane between 25–50% from the surface of the scaffold.

To stain for calcium deposits, 10-µm sections of scaffold were deparaffinised and stained with 2% filtered Alizarin red (Sigma-Aldrich) solution for 2 min. Sections were rinsed with distilled H$_2$O, dehydrated in xylene and mounted with DPX (a mixture of Distyrene, a plasticizer, and xylene). Alizarin red stained calcium deposits were then quantified by applying a 10% cetylpyridinium chloride (Sigma-Aldrich) solution for 5 min. The leached stain was then collected and plated on a 96 well plate before using a plate reader (Wallac 1420 Victor2 D, PerkinElmer, MA, USA) at 540 nm absorbance.

To determine the presence of phosphate-based mineral, 10-µm sections of scaffold were deparaffinised and stained by applying 2% silver nitrate (Sigma-Aldrich) solution for 1 h under bright light. The reaction was stopped by adding the developing solution, viz. 1% sodium thiosulphate (Sigma-Aldrich), for 1 min. Sections were dehydrated in xylene and mounted with DPX.

2.3.6. Statistics

Statistical analysis was determined using sigma statistical software package SigmaStat 3.0. The statistical differences between 2 groups were calculated using the Students t test and multiple groups were calculated using One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p < 0.05$. Results are presented as mean ± standard deviation.

3. Results

3.1. Scaffold fabrication

CollAqua slurries were initially fabricated using the standard operating procedure (SOP) for collagen GAG slurries (Haugh et al., 2011) with one of the three Aquamin variants, soluble, F or S, gradually added to the slurry. However, to improve the scaffold quality a number of parameters were adjusted including acetic acid molarity, addition rates and methods and the order in which they were added. Despite modifications to the protocols, it was not possible to produce good quality scaffolds at the higher Aquamin soluble and Aquamin S concentrations and as a result, these scaffold variations were not analysed further. In contrast, Aquamin F produced consistently high quality scaffolds at both 100 wt% (100 F) and 500 wt% (500 F) using the previously described method (Haugh et al., 2011) and these scaffolds were carried forward for analysis.

3.2. Material characteristics

3.2.1. Effect of Aquamin on mechanical properties

The mean compressive modulus for each scaffold type is shown in Fig. 1. The addition of Aquamin to the scaffolds resulted in significantly stiffer scaffolds at both 100 F (850 Pa) ($p < 0.05$) and 500 F (2700 Pa) ($p < 0.05$) when compared to collagen GAG alone (590 Pa). The 500 F scaffold was significantly stiffer when compared to the 100 F scaffold ($p < 0.05$).

3.2.2. Aquamin retention in the scaffold

Calcium release was used as a surrogate measure of Aquamin release from the scaffold. For each scaffold variant the maximum dry weight of calcium was calculated in the scaffold disk using the physical dimensions and calcium concentration. The calcium release from each scaffold was expressed as a percentage of the total calcium in the scaffold disk. No calcium ions were measured in any aqueous samples from the collagen GAG scaffolds (Fig. 2). In samples from the 100 F scaffolds, approximately 20% of the total calcium was released after just 1 min, this increased to
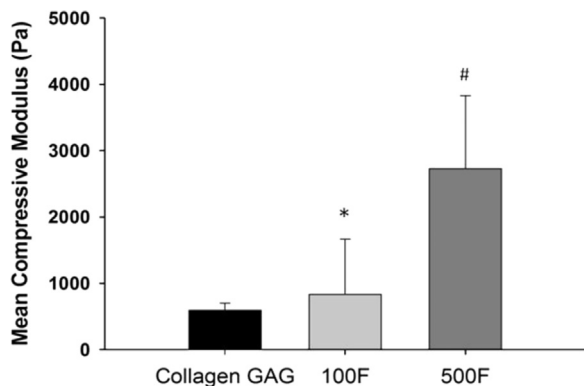
Fig. 1 – Scaffold compressive modulus (Pa) increases with the addition of Aquamin such that the 100 F is stiffer than collagen GAG alone (*$p < 0.05$) and the 500 F is significantly stiffer than the 100 F ($^{\#}p < 0.05$).
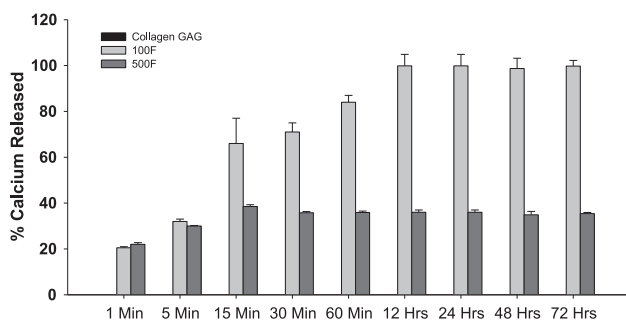
Fig. 2 – Calcium release from the collagen GAG, 100 F and 500 F scaffolds measured over a 3 day period. After 3 days, approximately 100% of the calcium in the 100 F scaffold was released while only 40% was released from the 500 F after the same time period.

approximately 30% after 5 min. A sharp increase, to greater than 60% release, was seen after 15 min with an 80% release measured after 1 h. By 12 h, all calcium within the scaffold was released. In contrast in the 500 F scaffolds, calcium release increased from 20% after 1 min to 40% after 15 min at which stage no additional calcium was released over the remaining 3 days.

3.2.3. Aquamin distribution within the scaffold

Aquamin distribution within the scaffolds was visualised using microcomputed tomography (microCT). As reported in previous studies, difficulties were encountered obtaining an image of the collagen GAG (Fig. 3a) scaffolds due to their low density (Al-Munajjed et al., 2009). The 100 F scaffolds (Fig. 3b) were also insufficiently dense to be distinguished from background noise by the microCT. As a result no difference was detected between the collagen GAG and 100 F scaffold types. A much higher quality image was obtained for the denser 500 F scaffold (Fig. 3c) showing a highly uniform distribution of material throughout the scaffold.

3.2.4. Effect of Aquamin on scaffold porosity and permeability

The results of the porosity calculations graphed in Fig. 4a show that Aquamin addition reduces porosity compared to collagen

GAG which has a porosity of 99.4%, however porosity remains above 95%. The 100 F scaffold was significantly less porous at 98.8% than the collagen GAG scaffold while the 500 F scaffold (96.7%) was significantly less porous than both the 100 F and collagen GAG scaffolds. No significant difference in permeability was measured between the 100 F (6.7×10^{-10} m^4/Ns) and 500 F (6.3×10^{-10} m^4/Ns) scaffolds (Fig. 4b). Although both Aquamin scaffolds had a significantly lower permeability than collagen GAG (1.6×10^{-9} m^4/Ns), the permeability remained high indicating good pore interconnectivity.

3.3. Biological characteristics

3.3.1. Effect of Aquamin on cell attachment

Cell number was determined using the Hoescht DNA assay. Using the initial cell seeding density and the calculated cell number from the assay, the percentage cell attachment was determined. The incorporation of Aquamin into the collagen GAG scaffold had no significant effect on cell retention by the scaffolds. At day 1, cell attachment was approximately 50% in all three groups, rising to 60% and 70% at days 3 and 7 respectively (not significant).

3.3.2. Effect of Aquamin on alkaline phosphatase protein expression

The osteogenic potential of the scaffolds was assessed using an ALP assay. At Day 1, there was no significant difference in ALP levels between the scaffolds. The results showed an increase in the alkaline phosphatase protein in the 500 F scaffold after 7 days when compared to the collagen GAG alone (Fig. 5). At days 21 and 28, ALP levels were significantly lower in 500 F than both 100 F and collagen GAG scaffolds. Protein levels between the collagen GAG and 100 F scaffolds was not significantly different.

3.3.3. Effect of Aquamin on osteopontin and osteocalcin protein expression

Osteopontin (OPN) and osteocalcin (OCN) are non-collagenous proteins that are produced by osteoblasts and are routinely utilised as bone formation markers and are indicative of osteogenesis. The collagen GAG scaffold is known to support osteogenesis and the presence of both OPN and OCN were observed at day 28 in the collagen GAG scaffolds. The incorporation of Aquamin at 100 wt% (100 F) into the collagen GAG scaffolds resulted in higher expression of both OPN and OCN at day 28 (Fig. 6). This expression was further increased in the 500 F scaffold where the highest expression of both proteins, but particularly OCN was observed.

3.3.4. Effect of Aquamin on mineralisation

Fig. 7 shows qualitative mineral deposition as measured by Alizarin red and von Kossa while Fig. 8 shows quantification of this Alizarin red staining. All cell-seeded scaffolds showed a cell mediated increase in Alizarin red staining from day 1 through day 28 with the greatest staining visible at day 28 in all groups. Comparison between the seeded collagen GAG, 100 F and 500 F scaffolds at days 21 and 28 identifies variations in calcium deposition between the scaffold types. At day 21, the 500 F seeded scaffold had significantly more calcium staining than the collagen GAG seeded scaffold. By day 28, staining in the
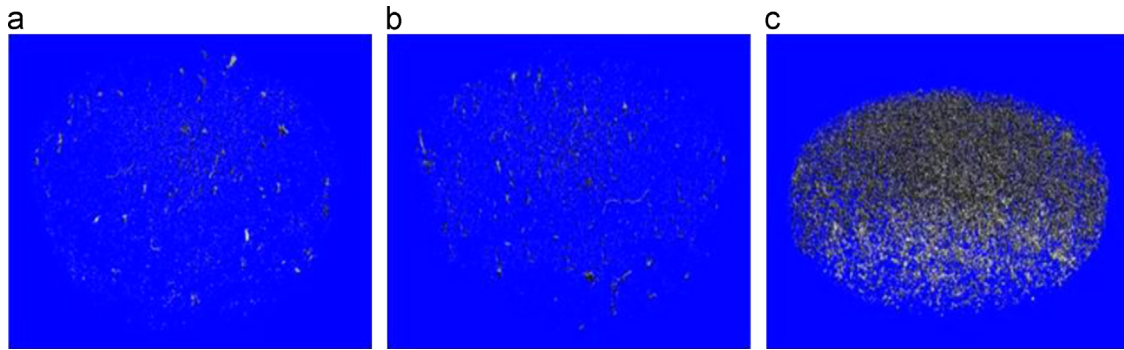
Fig. 3 – Calcium distribution within the scaffolds visualised using microcomputed tomography. No calcium can be seen in (a) collagen GAG or (b) 100 F. In (c) 500 F a homogeneous distribution of calcium can be seen.
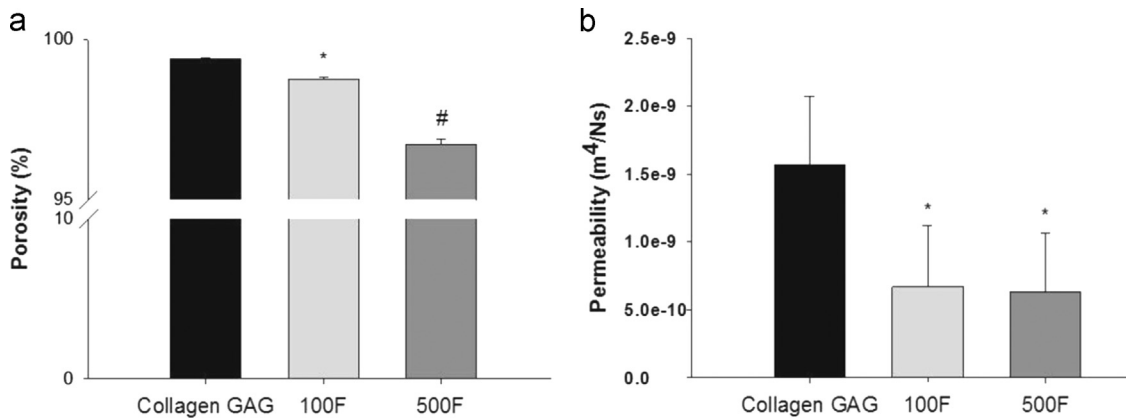

Fig. 4 – (a) A significant reduction in porosity is measured in the 100 F scaffold compared to collagen GAG alone (*$p < 0.05$). Addition of 500 wt% Aquamin produced a scaffold with a significantly lower porosity than both the 100 F and collagen GAG scaffolds (#$p < 0.05$) however porosity remained above 95%. (b) Permeability was significantly decreased after the addition of 100 wt% Aquamin (*$p < 0.05$). Further addition of Aquamin did not alter the permeability and 500 F scaffolds remained less permeable than collagen GAG.
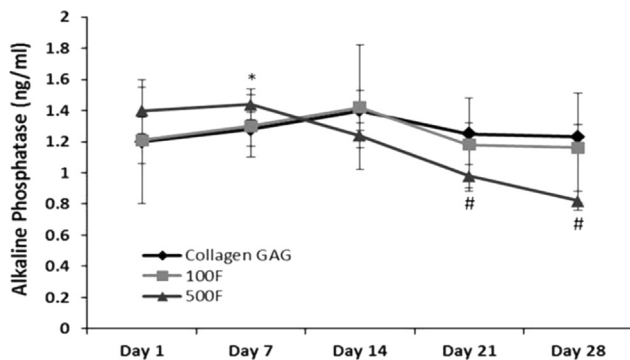

Fig. 5 – Alkaline phosphatase expression was significantly increased in the 500 F scaffold at day 7 when compared to collagen GAG (*$p < 0.05$). At days 21 and 28 alkaline phosphatase expression was significantly reduced in the 500 F scaffold when compared to expression in the collagen GAG and 100 F scaffolds (#$p < 0.05$).

500 F seeded scaffold had risen significantly to be greater than both the 100 F and collagen GAG seeded scaffolds. In the cell-free scaffolds, collagen GAG showed no Alizarin red staining. However, both the 100 F and 500 F cell free controls did show an increase in Alizarin red staining by day 28.

Examination of the cell-seeded von Kossa stained scaffolds (Fig. 7) showed similar results to the Alizarin red. Increased mineralisation was evident over time with the most staining in the seeded 500 F scaffold at days 21 and 28. No increase in mineralisation was observed in the cell-free control scaffolds, the significance of which is dealt within the discussion.

4.   Discussion

This study describes the successful development of a bone graft scaffold incorporating the multi-mineral Aquamin. The development of the scaffold was technically challenging yet resulted in a scaffold with improved osteogenic activity and enhanced mechanical properties whilst maintaining optimum porosity and permeability for successful bone tissue engineering. Furthermore, this work adds further credence to preliminary data (O'Gorman et al., 2012) which indicates the potential of Aquamin, a natural, organic, allergen free, plant-based mineral source to increase osteogenesis and improve bone formation.

Aquamin addition to collagen scaffolds resulted in a significant increase in the scaffold mechanical properties, specifically stiffness. The 100 F scaffolds were significantly
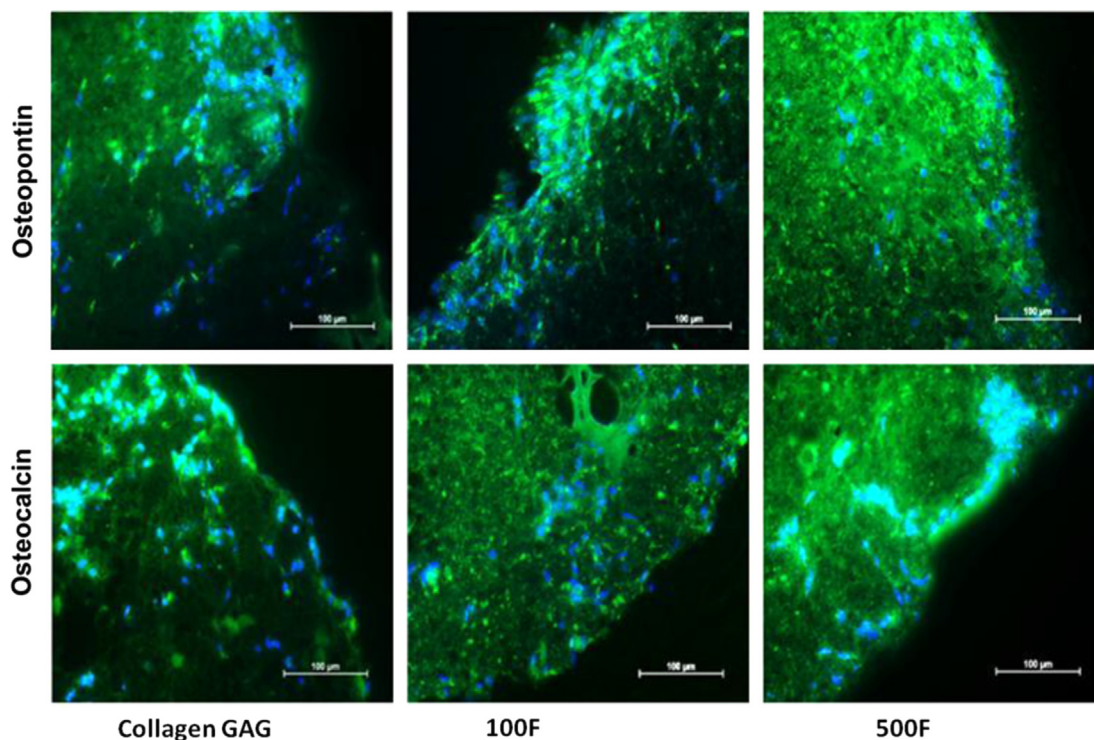
Fig. 6 – Osteopontin and osteocalcin protein expression (blue staining) were present in the collagen GAG scaffold at day 28. Protein expression was increased in the 100 F and 500 F scaffolds through the addition of Aquamin F where a positive correlation was seen between expression and Aquamin content. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stiffer than collagen GAG alone and the addition of 500 wt% Aquamin produced a 3-fold increase in stiffness. This increase in stiffness makes surgical handling of scaffolds easier. However, it also enhances the functional maturation of osteoblasts (Keogh et al., 2010). Numerous studies have shown that cells respond to the stiffness of the scaffold on which they are seeded. Comparison of matrix stiffness has shown that cells cultured on stiffer scaffolds tend towards osteogenesis rather than chondrogenesis.

The addition of Aquamin to the collagen GAG scaffold to produce the 100 F and 500 F scaffolds caused a decrease in porosity, which is important for cell infiltration and attachment and mass transfer of nutrients and wastes (Woodard et al., 2007). The least porous scaffold (500 F) still achieved porosities in excess of 95%, significantly above the minimum level suggested to facilitate cell infiltration and for bone repair (~90%) (Caliari et al., 2011). The porosity of the collagen GAG scaffold (99.4%) is in agreement with that reported in other studies (~99.5%) (O'Brien et al., 2004). Equally important, the permeability of the CollAqua scaffolds was such that it did not impede fluid flow through the scaffold demonstrating the high degree of interconnectivity of the pores within the scaffold. Despite the fact that the permeability of the 100 F and 500 F scaffolds was significantly less than the collagen GAG scaffold, the permeability remained within the range necessary for cells to penetrate the structure and for nutrients to diffuse (O'Brien et al., 2007). MicroCT analyses confirmed that the fabrication process produced a homogenous distribution of Aquamin within the 500 F scaffold. This distribution ensures an even release of Aquamin into the injury site.

One of the most interesting facets of the study came when the release kinetics of the CollAqua scaffolds was examined. The results demonstrated that approximately 80% of the calcium was released from the 100 F scaffold after 1 h with the remainder being released over the following 11 h. In contrast, the 500 F scaffold only released approximately 40% of its calcium after 1 h and no more release was observed from the scaffold for the remainder of the release study. The presence of the Aquamin in the 500 F scaffold makes it available to cells at a later time, thus having the potential to enhance osteogenesis over time (this is discussed later). This is likely due to the fact that Aquamin F is insoluble in water but soluble in acid. During slurry production, 2 M acetic acid is used which has the potential to solubilise the Aquamin. In the case of the 100 F scaffold, the volume of acetic acid was likely sufficient to solubilise all of the Aquamin. On exposure to water the solubilised Aquamin was released from the scaffold. In the 100 F scaffold all of the Aquamin was therefore released. In contrast, when fabricating the 500 F slurry there may be insufficient acetic acid to solubilise all of the Aquamin present. Therefore the 500 F scaffold instantly releases the solubilised Aquamin while the remaining insoluble fraction is retained and released more slowly from the scaffold. This type of sustained release is favourable to burst release in which the entire "active component" is released and subsequently degraded very rapidly.

The inclusion of Aquamin in the scaffolds produced an increase in osteogenesis. This was initially seen as increased alkaline phosphatase expression in the 500 F scaffold. ALP is a marker of matrix maturation and is not typically seen to
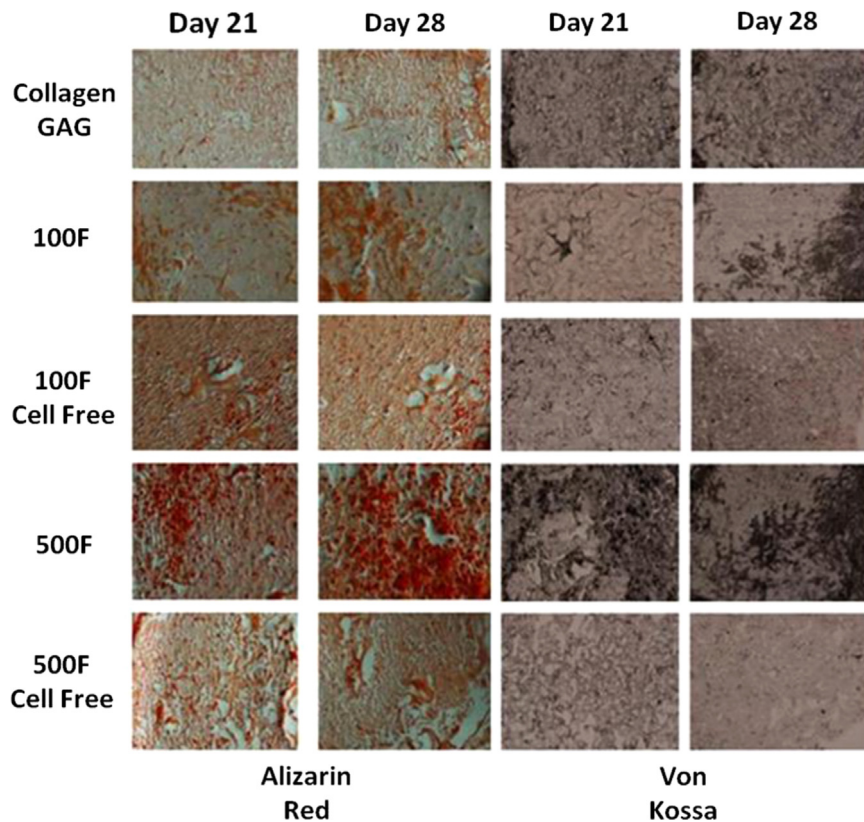
Fig. 7 – Effect of incorporation of Aquamin into a collagen-based scaffold on osteogenesis as assessed by mineralisation staining with Alizarin red and von Kossa. Increased staining of Alizarin red and von Kossa is evident at day 21 and day 28 in the 500 F cell seeded scaffolds. Increased Alizarin red staining was seen in the 500 F cell free control, however, a similar increase was not seen with von Kossa staining.
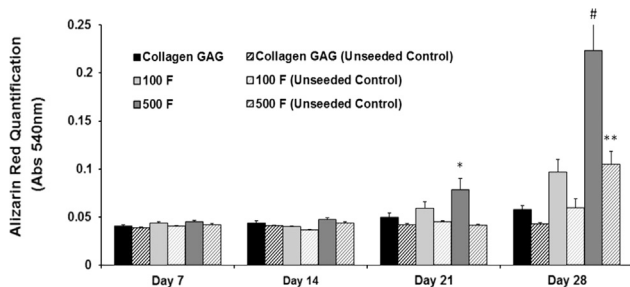
Fig. 8 – Alizarin red quantification measured a significant increase in staining by day 21 in the 500 F seeded scaffold in comparison to collagen GAG (*$p < 0.05$). At day 28, there was significantly more staining present in the 500 F than any other group ([#]$p < 0.001$). In the cell free 500 F control at day 28, staining was significantly greater than in either the 100 F or collagen GAG cell free controls (**$p < 0.05$).

express high levels in MC3T3 cells until much later (Stein and Lian, 1993). Expression usually peaks around day 21 before falling off again. However, in the 500 F scaffold, expression reached a maximum at day 7 after which it began to fall. Osteopontin and osteocalcin protein expression were also enhanced by the addition of Aquamin. Previously it has been identified that scaffold stiffness plays a role in increasing osteopontin and osteocalcin expression (Keogh et al., 2010;

Murphy et al., 2012). Thus, the stiffer Aquamin F scaffolds may also play a role in aiding osteogenesis.

We believe that the multi-mineral content in Aquamin, especially calcium, is the main contributor to the increased osteogenic expression. Calcium coated materials are believed to be good osteoinductors, as the composition of calcium aids in osteogenesis and bone development (Eyckmans et al., 2013). Osteoblasts can attach via N-cadherin, a calcium dependent cellular adhesive protein, which is an important regulator of osteoblast differentiation and osteogenesis (Marie, 2002). The additional mineral content in Aquamin also provides an explanation for the higher expression of osteopontin and osteocalcin in CollAqua scaffolds, however, further analyses would be necessary to confirm this.

The cell-free control scaffolds highlight some interesting results. Typically we measure mineralisation with Alizarin red or von Kossa stain. However, Alizarin red primarily stains for calcium which is the major constituent of Aquamin and thus a major component of the scaffolds. As was shown in the calcium release data, calcium is released from the Aquamin scaffold when it is placed in an aqueous medium. However, it was also shown that the 500 F scaffold retained approximately 60% of its calcium. When the Alizarin red stained scaffolds were examined, staining in the cell-free 500 F control was increased at days 21 and 28 relative to the earlier time points. This indicates that the calcium, which was up until this point contained in the scaffold, was

released and the Alizarin red stain can attach to it. This implies that we have an initial burst release of calcium within the first 2 h which is followed by a delayed release of calcium after approximately 21 days.

The highest levels of Alizarin red calcium staining were seen in the cell seeded 500 F scaffold after 28 days. The von Kossa phosphate stain was used to determine whether this mineralisation was a cell mediated response or as a result of the calcium being released from the Aquamin scaffold. Unlike calcium, phosphates are not naturally present in Aquamin and they will therefore not be present in the 500 F scaffold. The greatest level of von Kossa staining, and hence cell mediated mineral production, was in the 500 F scaffold at days 21 and 28. Thus, confirming Aquamin's ability to enhance osteogenesis and mineral production. However, we may not actually be seeing the true potential of Aquamin to promote osteogenesis and mineralisation in this study. During the cell culture feeds, spent media is removed and replaced with fresh media. Whilst this process removes waste material, it is also removing Aquamin which has been released from the scaffolds. Therefore, unlike in vivo where the Aquamin is retained in the body, in this situation the Aquamin is lost to the cells. An in vivo study evaluating Aquamin's potential to enhance bo9ne repair would be an interesting follow on study.

5.   Conclusions

This study provides further data to support the use of Aquamin to enhance bone quality and formation as it is clear that it has a significant pro-osteogenic effect on bone cells. A cell and growth factor free collagen-based bone graft substitute with enhanced mechanical properties over a collagen–GAG scaffold has been developed. The addition of Aquamin to the collagen–GAG biomaterial significantly improved mineralisation by osteoblasts and results in a new product which may be capable of enhancing osteogenesis to facilitate bone repair in vivo.

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