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Development and characterisation a collagen nano-hydroxyapatite composite scaffold for bone tissue engineering

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Abstract

Bone regeneration requires scaffolds that possess suitable mechanical and biological properties. This study sought to develop a novel collagen-nHA biocomposite scaffold *via* two new methods. Firstly a stable nHA suspension was produced and added to a collagen slurry (suspension method), and secondly, porous collagen scaffolds were immersed in nHA suspension after freeze-drying (immersion method). Significantly stronger constructs were produced using both methods compared to collagen only scaffolds, with a high porosity maintained (> 98.9 %). It was found that Coll-nHA composite scaffolds produced by the suspension method were up to 18 times stiffer than the collagen control (5.50 ± 1.70 kPa *vs*. 0.30 ± 0.09 kPa). The suspension method was also more reproducible, and the quantity of nHA incorporated could be varied with greater ease than with the immersion technique. In addition, Coll-nHA composites display excellent biological activity, demonstrating their potential as bone graft substitutes in orthopaedic regenerative medicine.

Keywords: Collagen, nanoparticles, hydroxyapatite, composite, bone tissue engineering

1. Introduction

Tissue Engineering (TE) is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that can replace, restore, or improve tissue function [1]. One approach of tissue engineering is to use 3-dimensional scaffolds to provide a suitable environment to induce tissue formation. Ideal scaffolds act as a guide supporting cell growth and differentiation and ultimately the deposition of regenerated tissue [2].

In bone tissue engineering, the scaffold should be biocompatible with osteoconductive and osteoinductive properties. The scaffold allows for cells to attach, proliferate and form extracellular matrix (ECM). It should have an open and interconnected pore structure (with a porosity >90 %) that allows nutrients to penetrate into the scaffold *in vitro* and then vascularisation to occur *in vivo* [3-7]. It should also degrade at a suitable rate to match the rate of tissue formation.

This paper focuses on the development of a novel composite scaffold for bone regeneration using the two major constituents of bone; collagen type 1 and hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) nanoparticles (< 100 nm). Collagen is used extensively as a scaffold biomaterial due to its biocompatible and biodegradable properties [8-12]. From an orthopedic perspective however, collagen scaffolds are limited by their poor mechanical characteristics and for this reason many studies, including research in our lab, have combined collagen with calcium phosphates to improve their mechanical properties [13-16]. However, poor resorbability and brittle constructs are problems that occur when using micron-sized HA particles [17, 18]. Therefore, the focus of this study was to develop a method of incorporating nano-sized HA particles (that have recently been optimized in our lab [19]) into highly porous collagen scaffolds based on the lyophilization technique [14] to produce collagen-nHA (Coll-nHA) biocomposite scaffolds with improved resorbability and mechanical characteristics.

2. Materials and Methods

2.1 Nano-hydroxyapatite (nHA) Synthesis

The nHA synthesis technique has recently been developed and optimized in our lab [19]. Briefly, nHA was produced by adding a solution of 6mM sodium phosphate tribasic dodecahydrate

($Na_3PO_4.12H_2O$), 7.5 mM NaOH (Sigma, Dorest) and 0.1 % Darvan 821A (R.T. Vanderbilt, Norwalk, CT) dispersant drop wise into an aqueous 10 mM solution of calcium chloride dihydrate (Fisher Scientific, Pittsburg) $CaCl_2$. A Ca/P ratio of 1.67 was maintained. The resulting precipitate (< 100 nm) was washed and resuspended using 10 mins of sonication.

2.2 Scaffold Fabrication

2.2.1 Collagen Control Scaffolds

Collagen control scaffolds were produced by freeze-drying a collagen slurry (0.5% (w/v) containing type 1 bovine collagen (Integra Life Sciences, Plainsboro, NJ) in 0.05 M glacial acetic acid [9, 20-22]. The slurry was freeze-dried in a stainless steel pan by cooling to -40˚C at 0.9˚C/min, followed by a sublimation step for 17 h at 0˚C and 200 mtorr (Advantage EL, Vir-Tis Co., Gardiner NY).

2.2.2 Composite Scaffold Fabrication

The Coll-nHA composites were synthesized using two different methods; (1) by adding the nHA particles to the collagen slurry prior to lyophilization (suspension method), and (2) by soaking collagen scaffolds into a nHA suspension (immersion method). In the suspension method, nHA particles suspended in water were added into the collagen slurry during the blending stage, followed by lyophilization. Two different concentrations of nHA suspensions were added (relative to weight of collagen used), 3.6 g nHA yielding a 100 % wt scaffold, *(S-100)* and 18 g nHA yielding a 500 % wt scaffold *(S-500).*

In the immersion method, 9.5 mm x 4 mm collagen scaffold discs were immersed in a nHA suspension for 4 days before being freeze-dried. The concentration of the calcium and phosphate solutions used were varied to generate two scaffold variants; calcium concentration 0.001 M *(I-low)* and 0.01 M *(I-high).* The Ca/P ratio for all suspensions was 1.67. All scaffolds were crosslinked and sterilized using a dehydrothermal treatment (0.05 bar at 105˚ for 24 hrs[14]).

2.3 Scaffold Characterisation

2.3.1 Mechanical Testing

Uniaxial compression testing was conducted to evaluate the effect of nHA incorporation on the Young's modulus of the composite scaffolds. All testing was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) fitted with a 5 N load cell. Samples were pre-hydrated in phosphate buffered saline (PBS) prior to, and during, testing. Compressive testing was performed at a strain rate of 10 % per minute. The compressive modulus was calculated from 2- 5 % of the stress-strain curve.

2.3.2 Fourier Transform Infra-Red spectroscopy

Fourier Transform Infra-Red (FTIR) spectra were collected to analyse and compare the material characteristics of all scaffold samples following nHA addition. FTIR analysis was carried out using a Spectrum One FTIR (Perkin Elmer, UK). Scaffolds were finely cut and mixed with potassium bromide before being pressed into a transparent sample. Spectra were collected between wavenumbers 4000 and 400 cm^{-1}.

2.3.3 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to examine the microstructure of the scaffolds. Scaffold samples were cut using a punch and fixed to an adhesive carbon stub. Imaging was carried out using a TableTop SEM (Hitachi High-Technologies Corp., Japan) operated at 15 kV.

2.3.4 Porosity

Porosity was calculated using the following equations:

$$\text{Porosity} = (\rho_{sample}/\rho_{material}) \times 100$$

where ρ_{sample} is the density of the sample and $\rho_{material}$ is the density of the material the scaffold is made from. For the composite scaffolds, $\rho_{material}$ is worked out based on the densities of collagen and nHA as follows:

$$\rho_{material} = m_{collagen} + m_{nHA} / V_{collagen} + V_{nHA}$$

where $m_{collagen}$ and $V_{collagen}$ is the mass and volume of collagen in the slurry respectively, m_{nHA} and V_{nHA} is the mass and volume of nHA in the slurry respectively.

2.4 *In Vitro* Analysis

2.4.1 Static Culture

Scaffold samples (ϕ 12.7 mm) were seeded with 2 x 10^6 MC3T3-E1 cells and cultured in media (DMEM supplemented with 10 % foetal bovine serum (FBS), 2 % penicillin/streptomycin and 1 % L-Glutamine (Sigma-Aldrich Ireland, Dublin)) to assess the behaviour of the composite scaffold following nHA addition (S-100) compared to the collagen only control.

2.4.2 DNA Quantification

A DNA Hoechst 33258 assay (Sigma-Aldrich, Germany) was used to evaluate cell number on the scaffolds following 7 days in culture by fluorescently labelling double-stranded DNA. Constructs were digested and homogenised in QIAzol lysis reagent (Qiagen Sciences, Maryland 20874, USA). Hoechst dye solution was then added to the digested samples and fluorescence was measured (excitation: 355 nm, emission: 460 nm) using a fluorescence spectrophotometer (Wallac Victor2, PerkinElmer Life Sciences). Readings were converted to cell number using a standard curve.

2.4.3 Histological Analysis

Wax embedded scaffolds were sliced (10 μm) and stained using Haematoxylin and Eosin (H&E) to reveal the distribution of cells throughout the construct.

2.5 Statistical Analysis

All data was analysed for significance ($p \leq 0.05$) using one-way analysis of variance (ANOVA) tests to compare group means. Post hoc tests to determine significant differences between group means were performed using the Tukey test.

3. Results

Collagen-nHA (Coll-nHA) composite scaffolds were successfully synthesised by using the suspension and immersion methods. Significant increases in modulus were achieved using both methods (Fig. 1), however both methods displayed a concentration effect *i.e.* higher moduli were

seen when higher concentrations of nHA were incorporated. The S-100 scaffold (100 % nHA added *via* the suspension method) showed no significant improvement *vs*. the collagen control, whereas the S-500 scaffold showed an 18 fold increase (5.50 ± 1.70 kPa *vs.* 0.30 ± 0.09 kPa) compared to it. Scaffolds made *via* the immersion method also showed improved compressive modulus values compared to the collagen control; at lower nHA concentrations a 2.5 fold increase was observed (I–low), and this was further improved (12 fold increase) when higher concentrations of nHA were used (I–high). This demonstrates the ability to tailor the mechanical properties of Coll-nHA scaffolds by varying the amount of nHA added using each method.

Fourier Transform Infra-Red (FTIR) spectra in Fig. 2 show the presence of nHA in the Coll-nHA composite scaffolds made by both methods (S-100 and I-High). Characteristic peaks for hydroxyapatite are located in the region of 500-600 cm^{-1}, the asymmetric bending and the stretching band of the $(PO_4)^{3-}$ group is found at 1063 cm^{-1}. In addition, characteristic peaks for collagen are seen at 2800-2950 cm^{-1} (C-H stretching), 1652 cm^{-1} (C=O group), and 3420 cm^{-1}(N-H stretching).

Fig. 3 shows the pore structure of the collagen control (Fig. 3a) and Coll-nHA composites made by the suspension (Fig 3b) and immersion techniques (Fig. 3c) using Scanning Electron Microscopy. The images show an open and interconnected porous structure with homogeneous pores for both the collagen control scaffolds and Coll-nHA composites. All scaffolds were found to be highly porous, retaining porosities above 98.9% (collagen control: 99.5 %, S-100 scaffold: 99.4 %, I-high scaffold: 98.9 %).

In vitro analysis was performed to assess the effect of the addition of nHA on biocompatibility. Upon addition of 100% wt nHA *via* the suspension method (S-100) there was no significant difference observed in cell number compared to the collagen control (Fig 4). Haematoxylin and Eosin (H&E) stained sections of the collagen control and (S-100) constructs revealed deep penetration of the MC3T3-E1 cells after 7 days in both constructs (Fig. 5). This verifies that the addition of nHA to collagen scaffolds does not have a detrimental effect on cell behaviour.

4. Discussion

The aim of this study was to develop and evaluate different methods of incorporating resorbable nHA particles (<100 nm) into collagen scaffolds for use in bone tissue engineering. Two novel methods were applied to produce composite scaffolds (a suspension method and an immersion method). The results show that the mechanical properties of the composites synthesized by each of the two methods could be tailored by varying the nHA content. The greatest increase in compressive modulus (18 fold *vs.* collagen control) was obtained by adding 500 % wt nHA using the suspension method. This result highlights the important mechanical role of the reinforcing ceramic material. Previous difficulties have been reported with increasing the filler content in composite constructs, often leading to irregular distribution of the ceramic and a reduction of the mechanical properties of the resultant scaffold (JH Song, Juhasz)[9, 23]. These problems have been overcome by using nano-sized particles and the novel approaches documented in this study, with up to 500 % wt nHA being incorporated homogeneously.

The variation of nHA content was more controllable using the suspension method, and higher quantities could be incorporated. There is an upper limit to the concentration of the nHA suspension used in the immersion technique, and hence to the amount of nHA that can be added in this way. This is due to larger particles forming during the synthesis at higher initial calcium and phosphate precursor concentrations, and these aggregates particles cannot penetrate into the porous collagen sponge during the immersion as required[19].

FTIR spectroscopy confirmed the incorporation of nHA into the scaffolds *via* both methods, while SEM demonstrated that all composites displayed an open and interconnected pore structure, similar to the collagen only control scaffold. Porosities were maintained above 98.9 % regardless of which method was used and the amount of nHA added. Higher magnification SEM images showed the formation of nHA aggregates (1-2 μm) in the scaffold immersed in higher concentration (I – high), which may be due to the long soaking period (4 days). Energy dispersive X-Ray analysis (EDX) substantiated the FTIR finding, confirming the presence of nHA in the Coll-nHA, with EDX results also demonstrating that a homogeneous distribution is achieved throughout the constructs. However, the immersed scaffolds were shown to also have a significant

level of NaCl present, which is due to the synthesis technique as this was not observed in the suspension scaffolds. For these reasons, scaffolds produced using the suspension method, (S-100), were selected to investigate the biological effect of the addition nHA to the biocompatible collagen scaffold.

H&E staining showed the widespread distribution of MC3T3-E1 cells throughout the scaffolds. The images showed that cells had migrated into the centre of the collagen control and Coll-nHA constructs (S-100) by 7 days, while the DNA assay revealed high cell numbers (*ca.* 2 x 10^6). This confirmed that the incorporation of nHA had no detrimental effect on osteoblasts, as scaffolds made by the suspension method displayed the same biological characteristics as the collagen only control. Therefore, the resorbable Coll-nHA scaffold demonstrated the same high biological activity as the collagen control scaffold, with considerably improved mechanical properties, validating its potential for bone tissue regeneration.

5. Conclusion

This study has led to the successful development of two novel techniques for the synthesis of resorbable Coll-nHA composite scaffolds with high biological activity. The suspension and immersion methods created significantly stiffer scaffolds with high degrees of porosity (< 98.9 %). Scaffolds made by the suspension method are more reproducible, less time consuming and the quantity of nHA added can be varied with greater ease than those produced using the immersion method, establishing them as potential bone graft substitutes in regenerative medicine.

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Fig. 1 Compressive strength of Coll-nHA composite scaffolds. Both methods show improved compressive moduli when compared to the collagen only control (* and ** denote significance $p < 0.05$)

Fig. 2 Fourier Transform Infra-Red (FTIR) spectra of collagen control scaffold, and Coll-nHA composites made by the immersion and suspension methods (S–100 and I–high) revealing the presence of nHA peaks in both composite scaffolds

Fig. 3 Scanning Electron Microscopy (SEM) images of (a) collagen control scaffold, (b) S-100 and (c) I-high composite scaffolds demonstrating the highly porous, interconnected structure of the new scaffolds comparable with the collagen control

Fig. 4 Graph showing cell number on collagen only control and Coll-nHA composite scaffolds (S-100) following 7 days in culture. There is no significant difference in cell number between the two groups, demonstrating that the high biological activity of the constructs is maintained following nHA addition

Fig. 5 Haematoxylin and Eosin stained transverse sections showing cell distribution after 7 days in the (a) collagen only control scaffold and (b) Coll-nHA composite scaffold (S-100) where cells have infiltrated into the centre of both constructs.