Mimicking Bone - Chemical and Physical Challenges

Sophie C. Cox
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Sophie C. Cox (WMG, University of Warwick)

Abstract

It is known that chemical and physical features of bone contribute to its functionality, reactivity and mechanical performance. This article presents a summary of previously published studies conducted by the author with the aim of fabricating a synthetic structure, referred to as a scaffold, which both chemically and physically emulates the intricate structure of bone. Novel work aimed at improving the understanding of the synthesis of a ceramic biomaterial, namely hydroxyapatite, that is chemically similar to bone mineral is discussed. A case study involving the manufacture of porous scaffolds by 3D printing is also presented. In summary, this article highlights a number of on-going challenges that multidisciplinary tissue engineers aim to solve to get one-step closer to mimicking bone, which clinically could improve the quality of life for millions of people worldwide.

Keywords: Bone tissue engineering; Scaffolds; Hydroxyapatite; Synthesis; Characterisation

Introduction

Bones perform several vital functions within the body, primarily structural support and protection of bodily organs. The ability of bone to self-repair and remodel to meet varying mechanical demands makes it a unique structural composite material (Chamay and Tschantz 1972; You et al. 2010). Bone also serves as (a) an attachment site for muscles to enable limb movement and joint mobility, (b) a reservoir for minerals (e.g. calcium and phosphorous), and (c) the primary site for the synthesis of blood cells.

The capacity of bone to function healthily can be affected by pathological conditions, diseases, and it is also well known that bone degenerates with age (Ritchie et al. 2006). Furthermore, the ability of bone to self-repair is limited by what is known as the ‘critical size defect’, defined by Schmitz and Hollinger as “the smallest intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal” (1986).

Major alterations in bone structure due to injury or disease can lead to discomfort and a reduced quality of life. Furthermore, defects outside of the limitations of natural self-repair may require surgical intervention, thus creating a demand for appropriate clinical strategies.

The highly organised and complex structure of bone, however, means this clinical need presents an on-going medical challenge. Traditionally bone grafts (BGs) and most commonly autografts – tissue harvested from another location in the patient’s body – have been used to fill or heal such defects. Despite being the ‘gold standard’, there are several disadvantages of autologous BGs, including painful harvesting surgery, limited supply, and long recovery times. These shortcomings have driven the biomedical research community to investigate alternative solutions that incorporate the use of synthetic biomaterials.

Specifically, an alternative strategy to traditional approaches is to create a temporary surrogate structure, which guides and encourages tissue regeneration. In order for such a strategy to be successful, it is necessary to combine expertise of cells, biochemical factors, and biomaterial science. This interdisciplinary field of research is known as tissue engineering and the structural component of this strategy is referred to as a ‘scaffold’.

Ideally, a scaffold should emulate the chemical and physical structure of the native tissue, thus it is crucial that an understanding of the properties that infer the functionality of bone is developed. Much attention has been given to calcium phosphate (CaP) based biomaterials since generally they are chemically similar to bone mineral. In particular, hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$ - HA) has been shown to exhibit a comparable crystal structure (Posner 1969, Cazalbou et al. 2004). This chemical likeness and the natural biological response that is elicited upon implantation is a key advantage of HA in comparison to other biomaterials commonly...
used in orthopaedic applications, such as metals (e.g. Ti6Al4V), polymers (e.g. HDPE) or bioglasses (e.g. 45S5). The Food and Drug Association (FDA), a USA agency that protects public health through supervision and regulation, has extensive guidelines for the in-vivo use of HA in the form of, for example, metallic implant coatings and void fillers.

This material, despite its promising features is known to exhibit poor resorbability and reactivity under physiological conditions compared to natural bone mineral due to its high stability (Cengiz et al. 2008). Substitution of trace elements that naturally occur in bone (for example Mg, Sr, Zn) and the use of nanosized (10⁹m) HA (nHA) have been shown to enhance the solubility, reactivity and response of bone cells to this synthetic material in-vitro and in-vivo (Webster et al. 2001; Leventouri 2006; Boanini et al. 2010). It is, however, important to note, that any changes to chemical composition must seek further FDA approval, which is a lengthy and costly process.

Much effort has been focused on the synthesis of this bioceramic due to the potential applications of HA as a bone replacement material (Elliott 1994; Raynaud et al. 2002; Bose and Saha 2003; Landi et al. 2008). The majority of such studies, however, are restricted to the preparation and structural investigation of HA without an evaluation of biological performance (Xue et al. 2006). Lack of biological testing is likely due to it being an expensive and time-consuming process. In addition, it may also be true, in some cases, that this is combined with a lack of appreciation of the effects of physicochemical alterations to the biological performance of HA.

Numerous authors have reported the fabrication of pure or composite HA scaffolds using a variety of techniques (Hutmacher 2000; Macchetta et al. 2009). In recent decades focus has been directed to the use of additive layer manufacturing (ALM) systems to produce such constructs layer-by-layer since they can be user defined, which inherently improves reproducibility and enables the creation of patient-specific products. It is relatively common that commercially purchased HA is used as a precursor material and as such the motivation of such studies is narrowed to the influence of physical attributes. That is despite the fact that the reactivity of bone mineral is largely determined by its composition and crystal structure, which in turn is determined by the synthesis method and reaction conditions (Cazalbou et al. 2004).

This article presents an overview of the author’s research to date while highlighting other key works within the field. The studies presented are focused on developing an
understanding of how the conditions used during aqueous precipitation (AP) of HA and 3D printing (3DP) influence critical scaffold properties. Ultimately, the aim of this article is to unearth chemical and physical challenges involved in mimicking bone and discuss the next steps of the author’s work as well as the field of biomaterials.

Chemical Challenges

The largest component of bone by weight is the mineral phase (65 – 70wt%), which can be described as a non-stoichiometric carbonated multi-substituted apatite that exhibits a similar crystal structure to HA. There are numerous conventional techniques that have been used to synthesise synthetic HA. A review of such methods is outside the remit of this article, however a comprehensive overview is presented in the author’s thesis (Cox 2013). This article is focused on synthesising HA via aqueous precipitation (AP), which is a popular method due to the use of relatively cheap raw materials and low temperatures resulting in minimal operating costs. AP reactions, however, cannot be deemed as trivial due to the simultaneous occurrence of crystal nucleation, growth, as well as coarsening and/or agglomeration. These underlying scientific mechanisms are not easy to differentiate and as a result reproducibility as well as the control of particle flocculation remain common factors for improvement (Narasaraju and Phebe 1996; Suchanek and Yoshimura 1998; Phillips et al. 2003). The sensitivity of phosphates and the need to fine tune the experimental conditions (e.g. pH, temperature) of AP reactions is reflected in the literature by the ranging phase purity, particle morphologies as well as sizes, crystallinity and thermal stability. Despite the shortcomings of AP methods, the potential to produce HA containing various ionic substitutions and its high scalability make it an attractive methodology, particularly for industrial scale production (Boanini et al. 2010). The AP method used by the author to synthesise samples A – G (Table 1) is outlined in Figure 1, for full details please refer to the referenced publications.
In spite of the widespread use of AP methods and the range of reaction conditions reported in the literature, few authors have systematically investigated the relationship between such conditions and biological performance. Novel work by Cox et al. showed through the use of in-vitro assays utilising MC3T3 osteoblast precursor cells, derived from mice, that changing the pH, temperature and solvent used during the AP of HA can significantly affect the degree of proliferation on such substrates (Cox 2013; Cox et al. 2014). Table 1 summarises the conditions that HA samples were precipitated under and results of the performed in-vitro assays. Fluorescence micrographs revealed that not maintaining the pH at 11 during synthesis (Sample A) resulted in dead (red) cells after 1 day of culture, illustrating the critical importance of the value and control of this parameter (Figure 2). A comparison of samples B and C, prepared under the same pH conditions but at 20 and 70°C, respectively, highlights that fine-tuning of AP reactions is vital to ensure a non-cytotoxic HA substrate is produced (Figure 2). Both of these differences in biological outcomes were attributed to subtle changes in physicochemical properties, which were identified through the combined use of a range of material characterisation methods, including surface area.

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*Figure 1: Flowchart illustrating the aqueous precipitation method used by Cox et al. to synthesise hydroxyapatite with varied conditions highlighted in red.*
analysis, zeta potential, as well as simultaneous differential thermal analysis (DTA) and thermogravimetry (TGA). In particular, differentiation of TGA curves (DTG) collected up to 1300°C revealed a significant change in the rate of weight loss for sample A between 200 and 300°C, which was not seen in samples B or C (Figure 3). Weight loss within this region was attributed to the dehydration of an acidic CaP phase, dicalcium phosphate dihydrate (CaHPO$_4$·2H$_2$O), otherwise known as brushite. Samples A and C were both shown to exhibit positive zeta potentials compared with the varying degrees of negative values measured for samples that were shown to be non-cytotoxic using a dead/live assay, such as sample B (Table 1). These positive values were attributed to the presence of secondary acidic CaP phosphates, which are proposed to have been released during culture resulting in cell death.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>C</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 2: Fluorescence micrographs from a dead/live assay illustrating the critical influence of pH and temperature on the viability of MC3T3 cells from 1 to 7 days of culture (red = dead; green = live) (Cox 2013)
Figure 3: Thermal graphs illustrating the influence of pH and temperature on the presence of secondary CaP phases in precipitated HA samples (black line = TGA; red line = DTA; blue line = DTG) (Cox 2013)
In addition to pH and temperature, the author has also previously investigated the effect of changing the solvent in which the HA crystals are precipitated. A proliferation rate of 194% between 1 and 7 days of culture of MC3T3 cells seeded on HA substrates precipitated in a mixed Toluene and deionised (DI) water solvent system was calculated from results of an in-vitro MTT assay (Table 1). This was compared with a 82% increase in cell number on HA prepared in the same manner but in a pure DI water system (Cox 2013). This significant increase in cell number was attributed to a reduction in the dielectric constant of the solvent system, as a result of using Toluene instead of polarised DI water, reducing the degree of crystallinity and surface charge as well as increasing the surface area of precipitated HA particles (Table 1).

The hexagonal crystal structure of HA enables partial or total replacement of ions, and in bone apatite numerous biologically relevant ionic substitutions occur. Divalent cations (e.g. Mg$^{2+}$) and monovalent cations (e.g. K$^+$) can substitute for Ca$^{2+}$, and anions such as fluoride (F$^-$) or chloride (Cl$^-$) may substitute for OH$^-$ groups. Furthermore, some ions such as carbonates can substitute for OH$^-$, PO$_4$$^{3-}$ or both, which are referred to as A, B and AB type substitutions, respectively (Cazalbou et al. 2004). Therefore, an appropriate formula for bone apatite can be expressed as (Ca, X)$_{10}$(PO$_4$, CO$_3$, Y)$_6$(OH, Z)$_2$ with X as substituting cations, and Y and Z being the substituting anions, with the indices 10, 6 and 2 changing according to the degree of stoichiometry (Weiner and Wagner 1998). In an effort to emulate the higher reactivity of bone mineral, a number of biomedical groups concerned with CaPs have investigated substituting naturally occurring ions into synthetic HA (Capuccini et al. 2008; Landi et al. 2008; Bohner 2009). A number of reports have confirmed that substitution of Sr, Mg or Zn can influence the crystallinity, solubility, surface charge, and physiological dissolution rate of synthetic CaPs (Boanini et al. 2010). Each of these divalent cations are known to play important roles in the biological responses of bone cells. Despite the synthesis of substituted HA attracting much interest, to the best of the author’s knowledge Sr, Mg or Zn substituted HA prepared under the same experimental conditions has not previously been reported. As highlighted above changes in reaction conditions, such as pH, can greatly influence the physical, chemical, and crystal structure of precipitated apatite, which makes it difficult to determine the sole influence of substituents when comparing literature since
variations in synthesis conditions, particularly pH and temperature, are common. Hence it is proposed that a comparison of HA doped with Sr, Mg or Zn prepared under the same experimental conditions is a more accurate assessment of the individual influence of these cations. Such a study was previously published by the author and concluded that doping HA with 10mol% Mg or 2mol% Zn enhanced the degree of cellular proliferation over a 7 day period compared with pure HA (Cox et al. 2014). The significantly higher proliferative rates calculated for Mg- and Zn-HA were attributed to the altered composition as a result of incorporation of dopants into the lattice structure and increased surface areas of particles (Table 1).

In summary, the author’s previously published work that is discussed provides evidence that cell proliferation may be positively influenced by maintaining a pH level of 11 during synthesis, precipitating HA in a mixed Toluene and DI water system, and doping HA with 10mol% Mg or 2mol% Zn. Generally, the selection of a synthesis method and conditions to produce HA cannot be deemed a trivial step in addressing the chemical challenge of mimicking bone mineral (Cox et al. 2014). In the words of Drouet, ‘all that glitters is not gold… all that is white is not apatite either’ (Drouet 2013).

Physical Challenges

Bone exhibits a heterogeneous and anisotropic structure that comprises different components at a range of length scales. Macroscopically bone is distinguished into cortical, otherwise known as compact, and cancellous, also referred to as trabecular or spongy bone. Cortical and cancellous bone can be easily distinguished by their degree of porosity: 4 - 28%, and 40 - 95%, respectively (Gibson 1985). The denser structure of cortical bone forms the outer region of all types of bone, the diaphysis (shaft) of long bones, and flat bones providing protection and support for the inner regions. In contrast, cancellous bone exhibits macro-sized pores, filled with bone marrow, which is found in the centre of all bones. The intricate organisation of bone confers its functionality, however, for biomedical scientists this precise structural arrangement
presents a number of challenges when trying to produce a scaffold that mimics its porosity and pore sizes, interconnectivity, topography, and mechanical strength.

Broadly, scaffold fabrication methods can be grouped as conventional or ALM techniques. In general, the fabrication method should adhere to: (1) does not adversely affect the chemical composition, mechanical properties or cytocompatibility of the material, (2) the technique should be accurate so pore size and morphology can be defined by the user, and (3) minimal variation in physical form between batches (i.e. consistency) (Leong et al. 2003). Scaffolds of complex shapes with a range of process dependent porosities from 30 - 98% can be produced by conventional techniques, such as freeze-drying or solvent casting. In contrast to ALM technologies, conventional processes require small capital input but typically scaffolds made using these techniques perform poorly mechanically in comparison to native bone. Reproducibility is another issue associated with these methods due to the inability to precisely control scaffold characteristics, such as pore size, pore interconnectivity and spatial distribution of pores. Conventionally fabricated constructs commonly fail to meet the demand to create highly porous networks necessary for cell growth, flow of nutrients and metabolic waste (Hutmacher 2000). Often, these techniques also require the use of organic solvents, such as chloroform, which if any residues remain in the structure may be toxic and/or carcinogenic to cells.

There are a variety of ALM systems based on computer aided design (CAD) and manufacture (CAM). Such techniques were first used for biomedical applications in the 1990s and can be categorised into three groups: (1) laser (e.g. stereolithography), (2) print or ‘ink’ (e.g. 3D printing) and (3) nozzle systems (e.g. fused deposition modelling). A comprehensive review of ALM techniques for the fabrication of bone scaffolds is presented by Hollister (2005). Generally, ALM techniques can be used to accurately fabricate parts of complex designs and near net shape processing minimises material waste. Furthermore, data generated from a scan (CT or MRI) of the patient may also be used as a template allowing the manufacture of customised implants, meaning desired levels of hierarchical complexity can be built into the part, which is advantageous when trying to mimic the intricate physical structure of bone. Due to the higher level of structural control it is possible to produce scaffolds via ALM techniques with superior mechanical properties in comparison to conventionally manufactured counterparts (Cox 2013).
The author’s work is focused on the use of an ALM technique called 3D printing (3DP), which is a powder based technology that involves layers of the stock material being bonded together by an appropriate liquid binder that is propelled onto the powder bed from a printer head. This process is much the same as what happens in an ink-jet printer but it is repeated layer-by-layer until the final 3D part is formed. Any unbound material may act as a support during the building process, however, this material must be removed after printing. In addition to exhibiting the inherent advantages of ALM systems, such as geometry control, 3DP creates parts with a rough surface. This is particularly advantageous for a bone tissue scaffold since the pits and troughs provide fixation points for cells to adhere to when it is implanted. Furthermore, the imperfect packing of powder particles results in small micropores within the solid structure, which may facilitate cell in-growth, vascularisation, and fluid flow.

For the 3DP process to work, it is essential that the powdered stock material has an ability to flow to enable adequate recoating of the part by the counter-rotating roller during layer-based manufacture. Particle size, morphology, and density have been reported to be critical factors in determining powder flowability (Butscher et al. 2012). A high level of flowability contributes towards an improvement in the resolution of the final part and vice versa. However, if the flowability is too high the powder bed can become unstable. Wettability of the powder particles by the binder solution is another crucial factor as it influences both resolution and mechanical strength of the 3D printed part. There are a number of parameters that influence powder wetting, including binder viscosity, topography of the powder bed surface (which itself is dependent on particle shape and size), and any chemical reactions between the binder and powder (Sachs et al. 1993).

Numerous authors have reported the fabrication of porous scaffolds by 3DP (Bose et al. 2013, Travitzky et al. 2014). Pure CaP powders, for example α- and β-tricalcium phosphate (TCP) (Butscher et al. 2012), tetracalcium phosphate (Khalyfa et al. 2007), and HA (Roy et al. 2003) as well as composites of CaPs mixed with organic polymers, such as poly(L-lactide-co-glycolide)-copolymer (PLGA) (Roy et al. 2003), have been utilised as stock materials. Material combinations that require the use of organic solvents as a binder, for example PLGA and β-TCP binded with chloroform

(Roy et al. 2003), have an inherent disadvantage as complete removal of the solvent is rather difficult due to the inherent porosity of 3D printed structures.

The feasibility of fabricating 3D porous scaffolds from powder compositions of HA and polyvinyl alcohol (PVOH), suitable for use as a component of the tissue engineering strategy has been reported by the author (Cox 2013). In short, to manufacture constructs purchased PVOH and HA powders were mixed together, compacted into the powder bed of a ZPrinter 310 (ZCorp, USA) and bound together using a commercially available binder (Zb90, ZCorp, USA) using a user defined layer thickness of 0.1mm. Post-printing, scaffolds were left to dry for 1hr before removing from the build bed, de-powdered using compressed air, dried in either a furnace or vacuum oven at 60°C for either 2 or 6hrs, and sintered at 1300°C. This study highlighted the potential of 3DP to produce constructs that exhibit key structural criteria, such as surface roughness and porosity (Figure 4), which are known to be vital in determining the success of bone tissue scaffolds. Figures 4c and d illustrate that designed pore channels and struts were accurately reproduced, this is important since the dimensions of these features were selected to enable cell migration and vascularisation. It is also significant that topographical features from the mm (the designed pores) to µm (surface topography) are present, since a range of features are required for different biochemical effects/functions. For example, protein interactions benefit from micron sized features, cellular development is facilitated by pores 1-20µm, bone in growth is improved by topographical pores 100-1000µm and implant functionality enhanced by pores >1000µm in size (Sanchez-Salcedo et al.2008).
Characterisation of powder flowability, assessed using a funnel flow method, strongly correlated with observed printability and can be deemed a vital prerequisite property since it influenced recoating of the build bed, which ultimately determined several critical physical criteria such as mechanical strength, microstructure, and porosity. Scaffolds produced from less flowable precursors (i.e. 60HA:40PVOH) were shown to be substantially weaker in compression and this was attributed to insufficient bonding between layers. Characterisation of green bodies (i.e. unsintered scaffolds) provided valuable information that facilitated an understanding of the shrinkage behaviour observed as a result of sintering to 1300°C. Significant differences in the size of micropores were observed between green scaffolds printed along the X and Y-axes, which resulted in variation of mechanical strength, and influenced the effectiveness of the removal of degradation products of PVOH during sintering.

Simply the more voids, or pores, within the structure the weaker it is. Drying of green bodies after printing was shown to improve the compressive strength of scaffolds by up to 350%; this was attributed to the shrinkage of micropores. Compression tests, performed on a 5800R 100kN static tester (Instron, UK) with 1kN load cell at a cross head speed of 10mm/min, also highlighted that interlayer bonding was critical to bulk strength and when parts were loaded perpendicular to the direction of printing (i.e. parallel to the boundaries of interlayer bonding) catastrophic failure (i.e. no or little plastic deformation) occurred. Highlighting that printing direction is a critical consideration to implant design. A maximum average compressive strength of 0.88±0.02MPa was exhibited by 50wt% HA green parts printed along the Y-axis and dried for 6hrs in a vacuum oven at 60°C, which is above the lower value of 0.15MPa reported for cancellous bone (Cox 2013).

Conclusions

Bone exhibits a highly intricate physical structure and complex chemical composition, which presents the multidisciplinary field of tissue engineering with a number of challenges when trying to produce synthetic scaffolds that emulate this remarkable composite. HA is a promising biomaterial for use in strategies to replace bone but our current understanding of this deceptive ceramic is not yet complete as highlighted by the author’s previous work, which is discussed in this article. The results of the synthesis studies referred to strongly advocate that pH should be maintained at 11 during preparation to ensure that a non-cytotoxic precipitate is formed. Furthermore, the potential advantages of using other solvent systems to water was highlighted by the promising cell studies conducted on HA synthesised in a mixed Toluene and water system. Overall, the author’s work in this area to date suggests that synthesis conditions must be carefully considered since they may have significant effects on cell proliferation in-vitro.

The 3D printed scaffolds illustrated in this article exhibit promising features for use in bone tissue engineering; rough topography for cell adhesion, compressive strength values comparable to cancellous bone, and an accurate replication of a user defined
an interconnected porous structure. Experimental work highlighted the importance of precursor characterisation and the advantages of printing along the same axis to part loading; in this case the Y-axis, as well as drying the parts in a vacuum oven for 6 hrs.

When considering the major advantage of ALM techniques to produce bone tissue scaffolds; the ability to physically tailor the construct, why should this idea not be combined with a chemically tailored stock material? The major shortcoming of the presented research is the challenges that the presented studies address have been achieved by focusing individually on either chemical or physical features. When looking at the author’s work as a whole it demonstrates the importance of investigating processing conditions so that techniques may be better understood and ultimately this may translate to improved patient outcomes. Overall, the results presented are the foundation of further work, which is being conducted to combine the outcomes of the aqueous precipitation and 3DP studies. In particular, scale up the reported HA synthesis method is being explored so that an adequate amount of enhanced powder may be produced to use in the 3DP process. If this is achieved, it will enable a new dimension of tailoring but while there are separate chemical and physical challenges, some of which are discussed here, there are certainly still many more difficulties to be unearthed in an attempt to catch up with evolution’s efforts. In conclusion, the research presented highlights the importance of multidisciplinary collaborations within the biomedical field, which may enable the next generation of customised implants to be created.

Acknowledgements

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References


Table 1: Summary of hydroxyapatite samples produced under varying conditions via aqueous precipitation

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Solvent</th>
<th>Ionic Substitution (mol%)</th>
<th>Surface Area (m²/g)</th>
<th>Zeta Potential (mV)</th>
<th>In-vitro assay results</th>
<th>Dead/Live</th>
<th>MTT (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10*</td>
<td>20</td>
<td>DI water</td>
<td>N/A</td>
<td>72.7±0.1</td>
<td>0.525</td>
<td>Dead (day 1)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>20</td>
<td>DI water</td>
<td>N/A</td>
<td>106.7±0.2</td>
<td>-21.3</td>
<td>Live (day 7)</td>
<td>82.8</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>70</td>
<td>DI water</td>
<td>N/A</td>
<td>132.3±0.7</td>
<td>2.98</td>
<td>Dead (day 3)</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>11</td>
<td>20</td>
<td>DI water (40 vol%) and Toluene (60 vol%)</td>
<td>N/A</td>
<td>126.1±0.6</td>
<td>-5.21</td>
<td>Live (day 7)</td>
<td>193.5</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>20</td>
<td>DI water</td>
<td>2 Sr</td>
<td>110.6±0.3</td>
<td>N/A</td>
<td>Live (day 7)</td>
<td>81.9</td>
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<tr>
<td>F</td>
<td>11</td>
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<td>DI water</td>
<td>10 Mg</td>
<td>170.8±0.8</td>
<td></td>
<td>Live (day 7)</td>
<td>161.9</td>
<td></td>
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<tr>
<td>G</td>
<td>11</td>
<td>20</td>
<td>DI water</td>
<td>2 Zn</td>
<td>117.1±0.2</td>
<td></td>
<td>Live (day 7)</td>
<td>124.3</td>
<td></td>
</tr>
</tbody>
</table>

*pH not maintained during synthesis; **proliferative rate over 7 days of culture
List of Figures

Figure 1: Flowchart illustrating the aqueous precipitation method used by Cox et al. to synthesise hydroxyapatite with varied conditions highlighted in red.

Figure 2: Fluorescence micrographs from a dead/live assay illustrating the critical influence of pH and temperature on the viability of MC3T3 cells from 1 to 7 days of culture (red = dead; green = live) (Cox 2013)

Figure 3: Thermal graphs illustrating the influence of pH and temperature on the presence of secondary CaP phases in precipitated HA samples (black line = TGA; red line = DTA; blue line = DTG) (Cox 2013)

Figure 4: 3D printed scaffolds manufactured from 50HA:50PVOH powders: a) Side view b) Top view, c) Designed pore channel, d) Scaffold strut, e) and f) Topography of scaffold surface (Cox 2013)