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COLLAGEN-HYDROXYAPATITE COMPOSITES FOR HARD TISSUE REPAIR

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Abstract

Bone is the most implanted tissue after blood. The major solid components of human bone are collagen (a natural polymer, also found in skin and tendons) and a substituted hydroxyapatite (a natural ceramic, also found in teeth). Although these two components when used separately provide a relatively successful mean of augmenting bone growth, the composite of the two natural materials exceeds this success. This paper provides a review of the most common routes to the fabrication of collagen (Col) and hydroxyapatite (HA) composites for bone analogues. The regeneration of diseased or fractured bones is the challenge faced by current technologies in tissue engineering. Hydroxyapatite and collagen composites (Col-HA) have the potential in mimicking and replacing skeletal bones. Both *in vivo* and *in vitro* studies show the importance of collagen type, mineralisation conditions, porosity, manufacturing conditions and crosslinking. The results outlined on mechanical properties, cell culturing and *de-novo* bone growth of these devices relate to the efficiency of these to be used as future bone implants. Solid free form fabrication where a mould can be built up layer by layer, providing shape and internal vascularisation may provide an improved method of creating composite structures.

Keywords: Collagen Type I, hydroxyapatite, composite scaffolds, biocompatible devices, bone substitute, tissue engineering

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Introduction

Bone tissue repair accounts for approximately 500,000 surgical procedures per year in the United States alone (Geiger *et al.,* 2003). Angiogenesis, osteogenesis and chronic wound healing are all natural repair mechanisms that occur in the human body. However, there are some critical sized defects above which these tissues will not regenerate themselves and need clinical repair. The size of the critical defect in bones is believed to increase with animal size and is dependent on the concentration of growth factors (Arnold, 2001). *In vivo* studies on pig sinus (Rimondini *et al.,* 2005) and rabbit femoral condyles (Rupprecht *et al.,* 2003) critical size defects of 6x10mm and 15x25mm respectively were measured. These defects can arise from congenital deformities, trauma or tumour resection, or degenerative diseases such as osteomyelitis (Geiger *et al.,* 2003). Bone substitutes allow repair mechanisms to take place, by providing a permanent or ideally temporary porous device (scaffold) that reduces the size of the defect which needs to be mended (Kohn, 1996). The interest in temporary substitutes is that they permit a mechanical support until the tissue has regenerated and remodelled itself naturally. Furthermore, they can be seeded with specific cells and signalling molecules in order to maximise tissue growth and the rate of degradation and absorption of these implants by the body can be controlled.

Bioresorbable materials have the potential to get round the issues that occur with metallic implants, such as strain shielding and corrosion. Titanium particles produced from wear of hip implants, were shown to suppress osteogenic differentiation of human bone marrow and stroma-derived mesenchymal cells, and to inhibit extra cellular matrix mineralisation (Wang *et al.,* 2003). Furthermore, these materials should help to reduce the problems of graft rejection and drug therapy costs, associated with for example the use of immunosuppressants (e.g. FK506) after implantation of bone grafts (Kaihara *et al.,* 2002).

When using a biodegradable material for tissue repair the biocompatibility and/or toxicity of both the material itself and the by-product of its degradation and subsequent metabolites all need to be considered. Further, at the site of injury, the implant will be subjected to local stresses and strains. Thus, the mechanical properties of the implant, such as tensile, shear and compressive strength, Young's modulus and fracture toughness need to be taken into consideration when selecting an appropriate material. However, given a bone analogue is ideally resorbable, these properties are not as important as for an inert implant which does not (intentionally) degrade. It is important for the bioresorbable material to be osteoconductive and osteoinductive, to guide and to encourage *de novo* tissue formation. The current aim of the biological implant is to be indistinguishable from the surrounding host bone

(Geiger *et al.,* 2003). It is self evident that creating new tissue will lead to the best outcome for the patient in terms of quality of life and function of the surrounding tissue.

Synthetic polymers are widely used in biomaterial applications. Examples in tissue engineering include aliphatic polyesters [polyglycolic acid (PGA) and poly-L-lactic acid (PLLA)], their copolymers [polylactic-co-glycolic acid (PLGA)] and polycaprolactone (PCL). However, the chemicals (additives, traces of catalysts, inhibitors) or monomers (glycolic acid, lactic acid) released from polymer degradation may induce local and systemic host reactions that cause clinical complications. As an example, lactic acid (the by-product of PLA degradation) was found to create an adverse cellular response at the implant site by reducing the local pH, in which human synovial fibroblasts and murine macrophages released prostaglandin (PGE_2), a bone resorbing and inflammatory mediator (Dawes and Rushton, 1994). Nevertheless, a potential way to stabilise the pH is by the addition of carbonate to the implant (Wiesmann *et al.,* 2004). Some polymeric porous devices also have the disadvantages of not withstanding crosslinking treatments such as dehydrothermal treatment (DHT) and ultraviolet (UV) irradiation (Chen *et al.,* 2001). The drawback of requiring chemical crosslinking (glutaraldehyde) is the formation and retention of potential toxic residues making these techniques less desirable for implantable devices (Hennink and van Nostrum, 2002). The reader is referred to Athanasiou *et al.* (1996) for a review in the biocompatibility of such polymeric materials.

Ceramics [eg HA, tricalcium phosphate (TCP) and/or coral] have been suggested for bone regeneration. Bone substitutes from these materials are both biocompatible and osteoconductive, as they are made from a similar material to the inorganic substituted hydroxyapatite of bone. However, the ceramic is brittle (K_c<1MPam^{-1}) (Dewith *et al.,* 1981) and does not match alone the mechanical properties of cortical bone (K_c~2-12MPam^{-1}) (Bonfield, 1984). Therefore, calcium phosphates have been used in areas of relatively low tensile stress such as bone/dental fillings or as coatings on implanted devices (Vallet-Regi and Gonzalez-Calbet, 2004).

Collagen, as a natural polymer, is increasingly being used as a device material in tissue engineering and repair. It is, for example, found in bone (Type I), cartilage (Type II) and in blood vessel walls (Type III) and has excellent biocompatible properties. Collagen is easily degraded and resorbed by the body and allows good attachment to cells. However, its mechanical properties are relatively low (E ~100 MPa) in comparison to bone [E ~2-50GPa (Clarke *et al.,* 1993)] and it is therefore highly crosslinked or found in composites, such as collagen-glycoaminoglycans for skin regeneration (O'Brien *et al.,* 2004), or collagen-hydroxyapatite for bone remodelling (Kikuchi *et al.,* 2004b). Both collagen and hydroxyapatite devices significantly inhibited the growth of bacterial pathogens, the most frequent cause of prosthesis-related infection, compared to PLGA devices (Carlson *et al.,* 2004).

Other approaches of bone repair have been to use autografts, allografts and xenografts. Although very successful in many operations, autografts have the disadvantages of insufficient supply and morbidity, as well as increasing surgery times and donor site pain (Uemura *et al.,* 2003). Allografts and xenografts are associated with infection and inflammation and have perceived ethical disadvantages. Xenografts also carry the risk of species-to-species transmissible diseases (Meyer *et al.,* 2004).

Careful consideration of the bone type and mechanical properties are needed for bone substitutes. Indeed, in high load-bearing bones such as the femur, the stiffness of the implant needs to be adequate, not too stiff to result in strain-shielding, but stiff enough to present stability. However, in relatively low load-bearing applications such as cranial bone repairs, it is more important to have stability and the correct three dimensional shapes for aesthetic reasons. One of many approaches of tissue engineering is to create a device of similar mechanical and biological properties to the one of the substituted tissue. Therefore, this review will focus on the engineering of a bone substitute, from the understanding of the individual and main components of bone to the creation of a collagen-HA composite. It will bring forward the idea that the manufacturing process of such biocompatible device defines its final microstructure, which in turn will determine its mechanical and biological response, and therefore its efficiency in repairing a hard tissue defect.

A Composite of Collagen and Hydroxyapatite

Skeletal bones comprise mainly of collagen (predominantly type I) and carbonate substituted hydroxyapatite, both osteoconductive components. Thus, an implant manufactured from such components is likely to behave similarly, and to be of more use than a monolithic device. Indeed, both collagen type I and hydroxyapatite were found to enhance osteoblast differentiation (Xie *et al.,* 2004), but combined together, they were shown to accelerate osteogenesis. A composite matrix when embedded with human-like osteoblast cells, showed better osteoconductive properties compared to monolithic HA and produced calcification of identical bone matrix (Serre *et al.,* 1993; Wang *et al.,* 1995). In addition, Col-HA composites proved to be biocompatible both in humans and in animals (Serre *et al.,* 1993; Scabbia and Trombelli, 2004).

These composites also behaved mechanically in a superior way to the individual components. The ductile properties of collagen help to increase the poor fracture toughness of hydroxyapatites. The addition of a calcium/phosphate compound to collagen sheets gave higher stability, increased the resistance to three-dimensional swelling compared to the collagen reference (Yamauchi *et al.* 2004) and enhanced their mechanical 'wet' properties (Lawson and Czernuszka, 1998). This happened even when the collagen was highly crosslinked.

Collagenase digestion can represent an *in vitro* measure of the rate of degradation and resorption of a biological implant. Uncrosslinked collagen and hydroxyapatite-collagen gel beads were analysed by collagenase digestion. HA-containing gel beads were less prone to collagenase and degraded more slowly than collagen gel beads. The

improved resistance of the composite material to degradation was explained by a potential competition of the hydroxyapatite to the collagenase cleavage sites, or by the absorption of some collagenase to the surface of HA (Wu *et al.,* 2004).

The direct comparison of other materials compared with Col-HA composites for bone substitutes have yet to be clearly investigated. However, increasing the biomimetic properties of an implant may reduce the problems of bacterial infections associated with inserting a foreign body (Schierholz and Beuth, 2001). Evidence of the biological advantage compared to artificial polymeric scaffolds have been further demonstrated in cartilage regeneration (Wang *et al.,* 2004). Polymeric scaffolds can take up to 2 years to degrade whilst Col-HA have a more reasonable degradation rate with regards to clinical use of 2 months to a year (Johnson *et al.,* 1996). Furthermore, osteogenic cells adhered better *in vitro* to collagen surfaces compared to PLLA and PGA implants (El-Amin *et al.,* 2003).

When comparing ceramic scaffolds and ceramic composite scaffolds, it was shown that Col-HA composites performed well compared to single HA or TCP scaffolds (Wang *et al.,* 2004). The addition of collagen to a ceramic structure can provide many additional advantages to surgical applications: shape control, spatial adaptation, increased particle and defect wall adhesion, and the capability to favour clot formation and stabilisation (Scabbia and Trombelli, 2004).

In summary therefore, combining both collagen and hydroxyapatite should provide an advantage over other materials for use in bone tissue repair. However, the manufacturing of such composites must start from an understanding of the individual components.

Collagen

The natural polymer collagen that represents the matrix material of bone, teeth and connective tissue can be extracted from animal or human sources. This may involve a decalcification, purification and modification process. This discussion will focus on collagen type I because it is by far the most abundant type used in tissue engineering and its use is widely documented (Friess, 1998).

Collagen type I: extraction from animal or human tissue

Skin, bones, tendons, ligaments and cornea all contain collagen type I. The advantage of using tendon or skin is that it eliminates the decalcification process of the bone mineral component. The removal of all calcium phosphate from a calcified tissue can be achieved through immersion in an Ethylenediaminetetracetic acid (EDTA) solution (Clarke *et al.,* 1993). This process can take as long as several weeks depending on the size of the specimen but it does not remove all antigens from the bone.

Collagen can be extracted and purified from animal tissues, such as porcine skin (Kikuchi *et al.,* 2004a) rabbit femur (Clarke *et al.,* 1993) rat and bovine tendon (Hsu *et al.,* 1999; Zhang *et al.,* 2004) as well as ovine (Damink *et al.,* 1996) and human tissue, such as placenta (Hubbell, 2003). The possible use of recombinant human collagen (although more expensive) could be a way of removing concerns of species-to-species transmissible diseases (Olsen *et al.,* 2003). Freeze- and air-dried collagen matrices have been prepared from bovine and equine collagen type I from tendons and the physical and chemical properties have been compared with regards to the potential use in tissue engineering scaffolds. The matrices of different collagen sources (“species”) showed no variations between pore sizes and fibril diameters but equine collagen matrices presented lower swelling ratio and higher collagenase degradation resistance (Angele *et al.,* 2004).

Collagen type I separation and isolation

The separation of collagen requires the isolation of the protein from the starting material in a soluble or insoluble form. Soluble collagen can be isolated by either neutral salts (NaCl), dilute acidic solvents (acetic acid, citrate buffer or hydrochloric acid) or by treatment with alkali (sodium hydroxide and sodium sulphate) or enzymes (ficin, pepsin or chymotrypsin) (Friess, 1998; Machado-Silveiro *et al.,* 2004). The addition of neutral salts can decrease protein solubility (*salting out*) (Martins *et al.,* 1998). The type of solvent required to isolate collagen will depend greatly on the tissue from which it is extracted, the amount of crosslinking present (maturity of the tissue) and whether decalcification is required. Other separation methods include gel electrophoresis (Roveri *et al.,* 2003) (SDS-PAGE and/or Western blotting). Collagen is then recuperated usually by centrifugation. Insoluble collagen can be isolated by modifying its structural configuration (mild denaturation agents) and by mechanical fragmentation (Friess, 1998).

Collagen modification and purification

Collagen type I can be modified chemically to achieve a polyanionic protein or a purified protein, known as atelocollagen. Polyanionic chemical modification can be achieved by selective hydrolysis of the asparagine (Asn) and glutamine (Gln) side chains of the collagen type I molecule and have the characteristic of having higher carboxyl group content (Bet *et al.,* 2001). Polyanionic collagen type I was found to improve cell adhesion by 1.5 times compared to native collagen type I (Bet *et al.,* 2003).

The purification of collagen is required to eliminate the antigenic components of the protein. These are mainly the telopeptide regions of collagen type I that can be most efficiently treated by enzymatic digestion. Pepsin is a widely used enzyme for the elimination and digestion of this immunogenic peptide (Rovira *et al.,* 1996; Zhang *et al.,* 1996; Hsu *et al.,* 1999; Kikuchi *et al.,* 2004a). As an example, rat tendon collagen type I was extracted and purified in 0.5mg/ml Pepsin in 0.5M acetic acid for 24 hours (Hsu *et al.,* 1999). However, complete immunogenic purification of non-human proteins is difficult, which may result in immune rejection if used in implants. Impure collagen has the potential for *xenozoonoses*, the microbial transmission from the animal tissue to the human recipient (Cancedda *et al.,* 2003). Furthermore, Wu *et al.* (2004) reported that pepsin treatment of impure collagen could

result in the narrowing of D-period banding. However, although collagen extracted from animal sources may present a small degree of antigenicity, these are considered widely acceptable for tissue engineering on humans (Friess, 1998). Furthermore, the literature has yet to find any significant evidence on human immunological benefits of deficient-telopeptide collagens (Lynn *et al.,* 2004).

Commercial collagen

Native collagen will have passed many extraction, isolation, purification, separation and sterilisation processes before they have been allowed to be used as biomaterial implants. Commercially available collagen type I can come either in insoluble fibril flakes (Sachlos *et al.,* 2003), in suspension (Muschler *et al.,* 1996; Miyamoto *et al.,* 1998; Goissis *et al.,* 2003), sheets or porous matrices (Du *et al.,* 1999; Du *et al.,* 2000). Many researchers use these collagens directly without further processing.

Hydroxyapatite Compound

Calcium phosphates are available commercially, as hydroxyapatite extracted from bones or they can be produced wet by the direct precipitation of calcium and phosphate ions.

Commercial Calcium Phosphate Powders

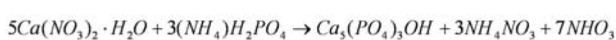
Hydroxyapatite powders can be obtained commercially with different crystal sizes. Unfortunately, such products may not be free of impurities. As examples, some commercially available HA particle sizes ranged between 10-40µm, averaged 5.32µm with a Ca/P ratio of 1.62 (Hsu *et al.,* 1999), while other sources had values of 160-200µm with a Ca/P ratio range of 1.66 to 1.69 (Scabbia and Trombelli, 2004). Most manufacturers produce sintered components which differ chemically from the biological carbonate apatites (Okazaki *et al.,* 1990). Sintering of HA (depending on stoichiometry) produces decomposition of the calcium phosphate phases to oxyapatite and possibly, tetracalcium phosphate and tricalcium phosphate (TCP). It has been found that stoichiometric HA is much less biodegradable than substituted HA and TCP (Kocialkowski *et al.,* 1990).

HA extraction from bone

Bone powders or hydroxyapatite have been extracted from cortical bovine bone (Rodrigues *et al.,* 2003). The bone was cleaned, soaked in 10% sodium hypochlorite for 24h, rinsed in water and boiled in 5% sodium hydroxide for 3h. It was then incubated in 5% sodium hypochlorite for 6h, washed in water and soaked in 10% hydrogen peroxide for 24h. The material was subsequently sintered at 1100°C and pulverised to the desired particle size (200-400µm). Grains of different crystal size could be separated by sieving. The final stages included sterilisation of the HA particles at 100-150°C.

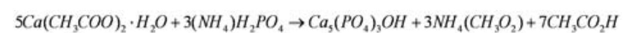
***In vitro* Hydroxyapatite (HA) powders**

Hydroxyapatite can also be precipitated *in vitro* through the following chemical reactions:



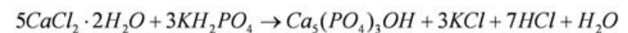
$$5Ca(NO_3)_2 \cdot H_2O + 3(NH_4)H_2PO_4 \rightarrow Ca_5(PO_4)_3OH + 3NH_4NO_3 + 7NHO_3$$

Ammonia was used to keep the solution basic (pH 12) (Sukhodub *et al.,* 2004). Hydroxyapatite precipitates were then extracted by heating the mixture to 80°C for about 10 minutes and incubating them at 37°C for 24h. Bakos *et al.* (1999) kept the reaction at pH 11, filtered the precipitate, washed it in distilled water and dried the solution at temperatures of 140-160°C. The dried material was then sintered at 1000°C for 2h before being crushed in a mortar. Only HA particles of 40-280µm were used for their composites. Alternatively, by using a different ammonium phosphate as a countercation for the phosphate ligand, non-stoichiometric hydroxyapatite powders have been filtered to an average particle size of 64µm, and then dried at 90°C. The cake is then ground and particles of 60-100 µm were used for the composites (Martins and Goissis, 2000).



$$5Ca(CH_3COO)_2 \cdot H_2O + 3(NH_4)H_2PO_4 \rightarrow Ca_5(PO_4)_3OH + 3NH_4(CH_3O_2) + 7CH_3CO_2H$$

The ceramic compound was synthesised at 60-80°C and at pH 7.4 (Okazaki *et al.,* 1990; Okazaki *et al.,* 1997). The apatite was then extracted by filtration, washed with distilled water and dried at 60-80°C. This method was further used to create $FgMgCO_3$Apatite for composite substitutes (Yamasaki *et al.,* 2003).



$$5CaCl_2 \cdot 2H_2O + 3KH_2PO_4 \rightarrow Ca_5(PO_4)_3OH + 3KCl + 7HCl + H_2O$$

In this reaction, chloride and potassium have been used as the counteranions and countercations respectively in order to form hydroxyapatite. As well as creating *in vitro* hydroxyapatite particles with controlled crystal size, this is a direct route for producing Col-HA composites by directly mineralising collagen substrates (Lawson and Czernuszka, 1998; Zhang *et al.,* 2004). The actual composite method of production will be reviewed later; however, it is important to be aware of differences in ion solutions used for the different experiments. For biomaterials, purity and sterility of all excipients is a key to favourable cellular response. Therefore, reaction 3 is recommended as it does not make use of calcium nitrate and ammonia. The purity of calcium nitrate was found to be directly linked to the purity of the precipitated calcium phosphate, whilst cytotoxic ammonia and its ammonium products are hard to remove (Kweh *et al.,* 1999).

Low temperature methods of HA processing have been proposed to avoid high crystallinity of HA due to sintering at high temperatures, resulting in similar carbonate substituted bone hydroxyapatite. These include colloidal processing, uniaxial and cold isostatic pressing, starch consolidation and a combination of gel casting and foaming (Vallet-Regi and Gonzalez-Calbet, 2004).

The influence of HA properties

HA implants or coatings are valuable because they provide a good adhesion to the local tissue due to their surface chemistry and have been shown to enhance osteoblast proliferation and differentiation (Xie *et al.,* 2004). In bone filling applications, bulk material is clinically harder to insert into a complex defect compared to injectable HA particles. Although particles provide an advantage of having a higher surface area, they are hard to manipulate alone and secure at the site of the implant. Therefore, they

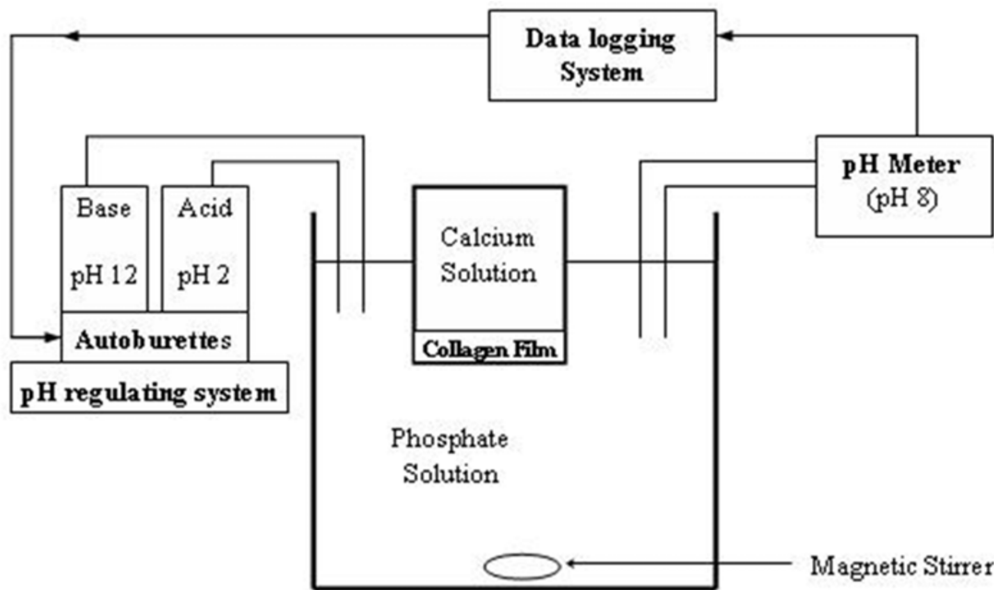


Figure 1. An experimental set-up for the direct mineralisation of a collagen sheet (Modified from Lawson and Czernuszka, 1998)

have been mixed with biodegradable matrices, such as collagen and PGA (Vallet-Regi and Gonzalez-Calbet, 2004).

The cellular response to HA particles, incorporated into a matrix or as coating, has been shown to depend on properties such as particle size and morphology (needle like, spherical or irregular plates), chemical composition, crystallinity and sintering temperatures. Due to such variability in HA properties, contradictions arise in the literature on the influence of one property over another and a need for a more systematic research was proposed by Laquerriere *et al.* (2005). However, it is generally accepted that needle shaped particles produce deleterious cellular response compared to spherical shaped particles. Indeed, macrophages have been found to release higher levels of inflammatory mediators and cytokines such as metalloproteinases (MMPs) and Interleukine-6 respectively (Laquerriere *et al.,* 2005).

In the case of collagen-HA implants, the size and crystallinity of the HA particles will have an importance to its stability and inflammatory response. In skeletal bones, carbonate substituted HA crystals are mineralised within small gaps of the collagen fibrils and have been quoted as 50nm×25nm×2-5nm in length, width and thickness respectively (Vallet-Regi and Gonzalez-Calbet, 2004). They provide a local source of calcium to the surrounding cells as well as interacting with collagen fibrils in order to achieve the relatively high mechanical properties of bones. However, small sintered particles of less than 1µm have been cautioned against in bone implants due to their increased inflammation response (Laquerriere *et al.,* 2005) and cell toxicity *in vitro* (Sun *et al.,* 1997). In contrast, Lawson and Czernuszka (1998) have shown that smaller plate-like particles of the order of 200nm×20nm×5nm produced an enhanced osteoblastic adhesion and proliferation compared to HA particles of an order of magnitude larger. These were carbonate substituted HA particles and produced at physiological temperatures (unsintered).

Collagen-Hydroxyapatite Composite Fabrication

This section will summarise the different methods for collagen-hydroxyapatite composite formation. It will include the production methods of composite gels, films, collagen-coated ceramics, ceramic-coated collagen matrices and composite scaffolds for bone substitutes and hard tissue repair.

***In vitro* collagen mineralisation**

Direct mineralisation of a collagen substrate involves the use of calcium and phosphate solutions. Collagen can either be a fixed solid film through which calcium and phosphate ions diffuse into the fibrils (Lawson and Czernuszka, 1998), or as a phosphate-containing collagen solution (Kikuchi *et al.,* 2004a), or an acidic calcium-containing collagen solution (Bradt *et al.,* 1999). The advantage of using the first method is that the orientation of the collagen fibres can be controlled (Iijima *et al.,* 1996). Indeed, it has been shown that the c-axis of HA crystals can be made to grow along the direction of collagen fibrils if the right conditions of mineralisation are met. These conditions (pH 8-9 and T =40°C) promote calcium ion accumulation on the carboxyl group of collagen molecules, leading to HA nucleation (Kikuchi *et al.,* 2004a).

The formation of HA is temperature and pH dependent as well as molar dependent (Ca/P ratio). Figure 1 shows an experimental set-up for calcium and phosphate diffusion and apatite crystallisation onto collagen substrates. Undenatured collagen films can be obtained from an acidic collagen suspension by air dehydration at different temperatures (4-37°C) or by applying cold isostatic pressure (200MPa) for 15h (Kikuchi *et al.,* 2001).

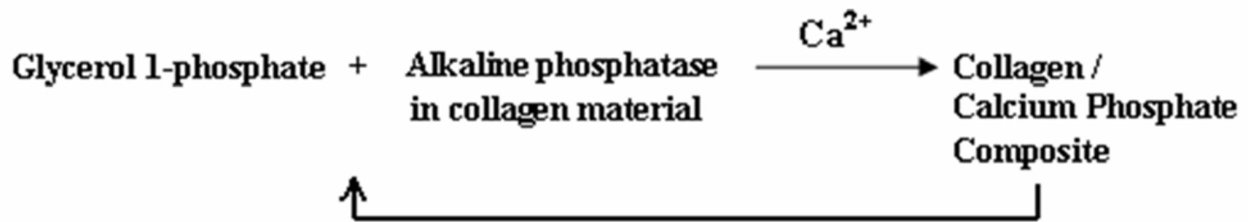


Figure 2. The cycle of enzymatic mineralisation of collagen sheets

Thermally-triggered assembly of HA/collagen gels

Liposomes have been used as drug delivery system due to their ability to contain water-soluble materials in a phospholipids layer (Ebrahim *et al.,* 2005). Pederson *et al.* (2003) have combined the direct mineralisation method with the ability of liposomes to exist in a gel for the potential use in injectable composite precursors. They reported a method whereby calcium and phosphate ions where encapsulated within the liposomes and the latter inserted into a collagen acidic suspension. After injection into a skeletal defect, the increase in temperature due to the body heat would start a gelation process, forming a collagen fibrous network where mineralisation occurs after reaching the liposome's transition temperature (37ºC) (Pederson *et al.,* 2003).

Vacuum infiltration of collagen into a ceramic matrix

Multiple tape casting is a method of ceramic scaffold production: an aqueous hydroxyapatite slurry containing polybutylmethalcrylate (PBMA) spheres is heated to high temperatures to burn out the BMPA particles, forming a porous HA green body (Werner *et al.,* 2002). Collagen infiltration was performed under vacuum, and the collagen suspension filled in the gaps of the porous matrix. The final composite was then freeze dried to create microsponges within. Variation of the final product was dependent on process time and flow resistance during filtration (Pompe *et al.,* 2003).

Enzymatic mineralisation of collagen sheets

Figure 2 shows a method of enzymatic loading of collagen sheets and the following cycle of mineralisation. The collagen containing an alkaline phosphatase was allowed to be in contact with an aqueous solution of calcium ions and phosphate ester. The enzyme provided a reservoir for PO_4^{3-} ions for calcium phosphate to crystallise and mineralisation was found to occur only on these coated areas (Yamauchi *et al.,* 2004). The sample was then coated again with a collagen suspension, air dried and cross-linked with UV irradiation. Repeating this cycle resulted in multilayered composite sheets of calcium/phosphate and collagen, with a sheet thickness of 7µm (Yamauchi *et al.,* 2004).

Water-in-Oil emulsion system

Col-HA microspheres or gel beads have been formed in the intention of making injectable bone fillers. A purified collagen suspension mixed with HA powders at 4°C was inserted in olive oil and stirred at 37°C. The collagen aggregated and reconstituted in the aqueous droplets. The phosphate buffered saline (PBS) was added to form gel beads (Hsu *et al.,* 1999). However, this method has the disadvantage of not being able totally to remove the oil content from the composite. Additionally, composite gels for injectable bone filler have the disadvantage that the viscosity of the mixture becomes too low on contact with body fluids, resulting in the "flowing out" from the defect (Kocialkowski *et al.,* 1990).

Freeze Drying and Critical Point Drying Scaffolds

In order to form a sponge-like porous matrix, either freeze-drying or critical point drying (CPD) is required. A collagen/HA/water suspension can be frozen at a controlled rate to produce ice crystals with collagen fibres at the interstices. In the case of freeze-drying, ice crystals are transformed to water vapour at a specific temperature and pressure by sublimation. In the case of CPD, liquid and vapour become indistinguishable above a certain pressure and temperature, where both densities of the two phases converge and become identical (supercritical fluid shown in Figure 3). Above this critical point, surface tension is negligible resulting in little matrix collapse. The lower critical point of carbon dioxide (31.1°C, 7.3MPa) compared to water (364°C, 22.1MPa) makes the use of CO_2 very popular when critical point drying.

Freeze drying and critical point drying have the fewest residual solvent problems compared to other scaffold manufacturing techniques. Furthermore, the easy removal of ice crystals compared to porogens, used in conventional

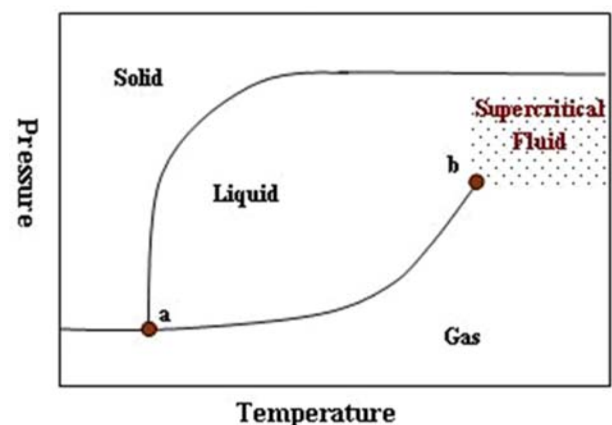


Figure 3. Phase Diagram of Carbon Dioxide. (a) Triple Point (-56.4ºC and 0.5MPa) (b) Critical Point (31.1ºC and 7.3MPa).

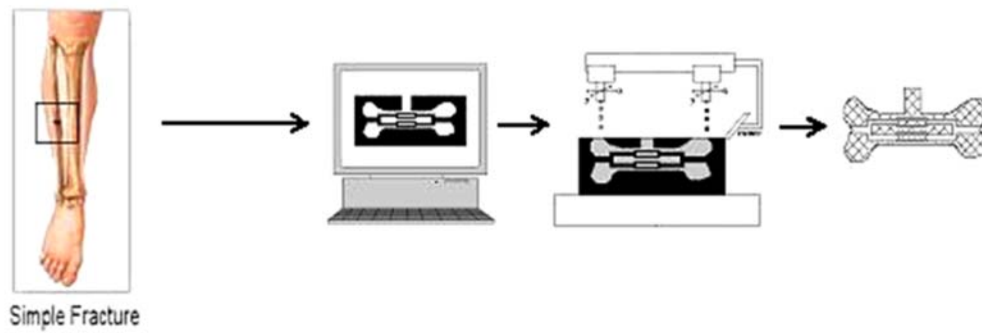


Figure 4. The use of Solid Freeform Fabrication in the design of composite scaffolds

polymeric-porogen leaching, eliminates any dimensional restrictions (Leong *et al.,* 2003). In freeze drying and critical point drying processes, pore sizes are determined by the ice crystal formation. Changing the freezing rate and solubility of the suspension as well as the collagen concentration can alter the pore size. Additional solutes (ethanoic acid, ethanol) can create unidirectional solidification of collagen solutions (Schoof *et al.,* 2000), and lower freezing rates generate larger pore sizes (O'Brien *et al.,* 2004). Pore size is important in scaffolds as they will determine cell adhesion and migration, the mechanical properties of the scaffold and as a result the success of new tissue formation. Karageorgiou and Kaplan (2005) recommended biomaterial scaffolds to possess pore sizes of over 300µm in order to favour direct osteogenesis and to allow potential vascularisation.

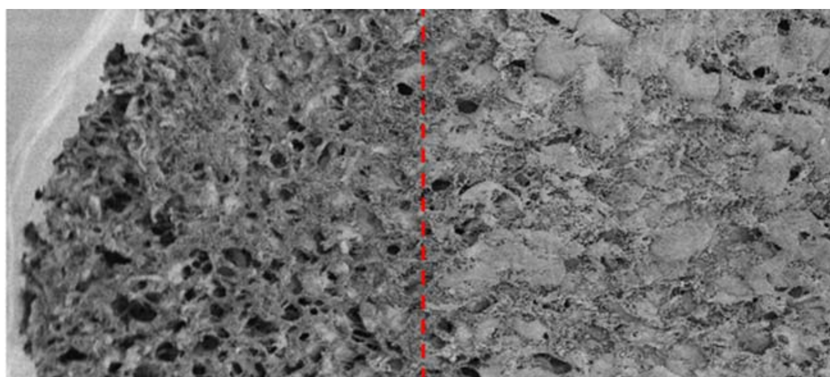
Solid Freeform Fabrication with Composite Scaffolds

Figure 4 shows a schematic of a computer model of a bone, to the creation of a mould (3-D printing) and to the scaffold production. The model is first drawn with the help of computer aided design, the mould is then printed with a "support" and "build" material, the sacrificial mould dissolved to obtain the casting mould, Col-HA cast into the mould and frozen, ice crystals replaced with ethanol, ethanol-liquid CO_2 exchanged and critical point dried to finally arrive with an exact porous replica of the original design. This method has been used extensively by Sachlos (Sachlos and Czernuszka, 2003; Sachlos *et al.,* 2003) and is part of many solid freeform fabrication or rapid prototyping methods used to form scaffolds for tissue engineering and reviewed by Leong *et al.* (2003).

Solid freeform fabrication techniques have recently been developed with artificial polymers and ceramic materials (Taboas *et al.,* 2003; Hutmacher *et al.,* 2004). These have the ability to change pore interconnectivity, pore size and pore shape, but have the disadvantage of not having the affinity of collagen to cell attachment. Another major advantage of Collagen-HA scaffolds produced through the SFF method is the ability to control variables at several length scales:

Control of the external structure: computerised tomography (CT) or magnetic resonance imaging (MRI) scans can be used to engineer biocompatible scaffolds. Although CT scans are 2-dimensional and MRI scans less sensitive to skeletal tissues (bones), it is possible to obtain the relative dimensions of a defect. These scans can be directly converted to a computer design and then the mould or scaffold itself printed out with solid freeform fabrication techniques.

Control of the internal structure: the Harvesian system of bone is a very complex system of vascularisation, in which cells are not found beyond 200µm of a blood supply (and therefore oxygen). 3-D printing can incorporate such architecture in its scaffold manufacture, with the hope that



2 mm

Collagen 1wt%	Collagen 5wt%
Mean pore diameter: 70µm	Mean pore diameter: 15µm

Figure 5. Scanning Electron Microscopy Image of a collagen scaffold with graded porosity. On the left of the scaffold, the mean pore diameter is ~70µm, and on the right the mean pore diameter is ~15µm. This type of scaffold could be used to engineer hybrid tissues.

Table 1. Common physical and chemical methods of crosslinking collagen

Material	Crosslinking method	References
Physical Treatment		
Dermal bovine collagen	DHT at various temperatures and vacuum	(Gorham *et al.*, 1992)
Dermal bovine collagen	UV irradiation at various wavelength, time and atmosphere	(Miyata *et al.*, 1971) (Torikai and Shibata, 1999)
Chemical Treatment		
Collagen based scaffolds	Diphenylphosphorylazide (DPPA)	(Vaissiere *et al.*, 2000) (Roche *et al.*, 2001)
Dermal sheep collagen Pericardium bovine collagen	1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC)	(Damink *et al.*, 1996) (Lee *et al.*, 1996)
Dermal sheep collagen Dermal porcine atelocollagen	Glutaraldehyde (GA)	(Damink *et al.*, 1995) (Kikuchi *et al.*, 2004b)
Crosslinking comparison		
Dermal bovine atelocollagen	Dehydrothermal treatment (DHT) and UV-irradiation	(Suh *et al.*, 2001b)
Collagen based films and scaffolds	GA, EDC, acyl azide-based (DPPA) and cyanide-based chemicals	(Rault *et al.*, 1996)
Dermal bovine collagen films	UV irradiation, DHT, GA, carbodiimide	(Weadock *et al.*, 1983)

neo-vascularisation (angiogenesis) will form after scaffold implantation.

Control of porosity: The freezing rate and the collagen/HA content are the key factors in controlling pore size. The composite scaffolds can therefore be tailored to have certain porosities favourable to cellular adhesion and migration. Figure 5 shows the possibility to adjust porosity within regions of an individual scaffold.

Control of crosslinking: Chemical and physical crosslinking are additional means of affecting the mechanical properties and the degradation rate of the scaffolds. Table 1 lists the most common treatments currently in use for collagen crosslinking. Collagen fibres which have been chemically crosslinked have a risk associated with the potential toxicity of residual molecules or compounds after implantation (Friess, 1998). Therefore, physical crosslinking by thermal dehydration at 110ºC under vacuum or by a controlled exposure to ultraviolet light have been proposed as having less risk, but have the drawback of limited crosslinking and potentially causing partial denaturation of collagen. These crosslinking methods have been shown to increase the Young modulus, the swelling resistance and resistance to enzymatic digestion (Weadock *et al.,* 1983) and provide additional ways for tailoring the properties of a scaffold.

Commercially available composite

The commercialisation of Col-HA composites for hard tissue repair means that these have met clinical health and safety requirements. Table 2 summarises some of the available composites on the market today.

Collagen-Hydroxyapatite Composite Comparison

This chapter will discuss results obtained from different manufacturing routes with regards to the mechanical properties and the *in vivo* and *in vitro* cellular response of Col-HA composites.

Mechanical properties: Ultimate Tensile Strength (UTS) and Young's Modulus (E)

Animal skeletal bones have been shown to exhibit mechanical properties as high as 2-50GPa for elastic modulus and 40-200MPa for ultimate tensile strength (Clarke *et al.,* 1993). As seen in Table 3, the influence of one type of composite manufacturing compared to another on the implant's mechanical properties is difficult to assess, as many variables come into play. The variation in the ratio of collagen to hydroxyapatite, the degree of porosity of the composite and the degree of crosslinking involved in Modulus (E) and Ultimate Tensile Strength (UTS) of the specimens. Currey (1999) demonstrated when assessing different animal hard tissues, that in general, a high mineral

Table 2. Commercial collagen-hydroxyapatite composite

Composite	Name (Company)	Manufacturing
Synthetic HA Bovine Collagen type I (calf skin)	Collapat II (BioMet, Inc)	Direct Mix Freeze dried
Synthetic HA Bovine Collagen type I Bone marrow aspirate	Healos (Depuy Spine, Inc)	HA-coated collagen matrices
Biphasic HA/TCP Bovine Collagen type I Autologous bone marrow	Collagraft (Neucoll, Inc; Zimmer, Inc)	Composite Paste (direct mix of equal mass proportions) with a 4:1 volume ratio of autologous bone marrow aspirated from the illium. Available in a lyophilised Composite Strip
Synthetic HA Equine Collagen type I Chondroitin-6-Sulphate (CS)	Biostite (Vebas, s.r.l)	Freeze Dried HA (88wt%), Col (9.5wt%), CS (2.5wt%)

Table 3. Mechanical properties of different Col/HA scaffolds. (N/M= Not Measured, *= no Glutaraldehyde)

Method	Calcium Phosphate (wt%)	Porosity	Crosslinking	UTS (MPa)	E (GPa)
Enzymatic mineralisation (Yamauchi *et al.*, 2004)	N/M	~ 0 air dried	UV-irradiation	45-60	2-3
Direct mineralisation (Lawson and Czernuszka, 1998)	0-39	~0 Collagen film	No	34-53	0.44-2.82
Mineralisation of decalcified bone (Clarke *et al.*, 1993)	10-15	N/M	No	44.87	0.68
Direct mineralisation (Du *et al.*, 2000)	60-70	Porous Freeze-dried	No	6-11	0.66-2.24
				Bending Strength (MPa)	
Direct mineralisation (Kikuchi *et al.*, 2004b)	80	~ 0 Pressurised	Glutaraldehyde	7.5* 22	N/M
Direct mix (Bakos *et al.*, 1999)	90	Porous Lyophylised collagen matrix	No	3.58 kPa	N/M

Table 4. A comparison of *in vitro* and *in vivo* experiments using different formulations of Col-HA composites

Composites	Method	Cell culturing or implantation	Period (new tissue formed)
In vitro experiments			
HA-Collagen (Du *et al.*, 1999)	*In vitro* mineralisation Composite porous sheets	Bone extract wrapped around composite Osteogenic cells	21 days (mineralised bone)
HA-Collagen (Clarke *et al.*, 1993)	Remineralisation of decalcified bone	Human derived bone cell culture	32 days (connective tissue and mineralised bone)
HA-Collagen (Wu *et al.*, 2004)	Water-in-Oil emulsion Composite gel beads	Osteoblasts from rat calvaria	21 days (mineralised bone)
HA-Collagen (Wang *et al.* 2004)	--- Porous composite	Chondrocytes in closed chamber	49 days (cartilage)
HA-Collagen-Elastin (Rovira *et al.*, 1996)	Direct mix, air dried Low porosity	Osteoblasts from trabecular bone	15 days (mineralised bone)
In vivo experiments			
$C0_3$Apatite-Collagen (Okazaki *et al.*, 1990)	Direct mix, centrifuged, air dried. Low porosity.	Implantation in rat periosteum cranii	21 days (mineralised bone)
PCCA-TCP-Collagen (Du *et al.*, 2000)	*In vitro* mineralisation Composite porous sheets	Implantation in rat thigh muscle	7 days (connective tissue and capillary vessels)
HA-Collagen (Kikuchi *et al.*, 2001)	*In vitro* mineralisation Dense cylinders with internal tunnels	Implantation in beagle tibia	54 days (mineralised bone)
HA-Collagen (Itoh *et al.*, 2002)	*In vitro* mineralisation Dense composite	Implantation in beagle cervical spine	14 days (start of callus formation)
$FGMgCO_3Ap$ – Collagen (Yamasaki *et al.*, 2003)	Direct mix, air dried	Implantation in rat periosteum cranii	14-28 days (mineralised bone)
HA-Collagen Alginate (Sotome *et al.*, 2004)	*In vitro* mineralisation Freeze dried	Implantation in rat femur	14 days (mineralised bone)
nHA-Anionic Collagen (Martins and Goissis, 2000)	*In vitro* mineralisation Composite paste	Implantation in rat infraorbital bone	60 days (mineralised bone)
cAP- Collagen (Suh *et al.*, 2001a)	Direct mix, freeze dried High porosity	Implantation in rabbit skin	98 days (mineralised bone)

content combined with low porosity exhibited higher UTS and E, expressing the greater importance of the ceramic component.

The strengthening effect of HA can be explained by the fact that the collagen matrix is a load transfer medium and thus transfers the load to the intrinsically rigid apatite crystals. Furthermore, the apatite deposits between tangled fibrils 'cross-link' the fibres through mechanical interlocking or by forming calcium ion bridges, thus increasing the resistance to deformation of the collagenous fibre network (Hellmich and Ulm, 2002). The mechanical properties of all the tabulated composites are lower than the natural properties of bones. The highly organised structure of cortical and cancellous bones is very hard to reproduce *in vitro*, and more research is needed on improving the Col-HA composites if they properly want to imitate skeletal bone structure.

Cell culturing and *in vivo* implantation of composites

Results of using collagen-calcium phosphate composites *in vitro* (using osteogenic cells) and *in vivo* (in bone defects) are presented in Table 4. The table illustrates the potential for fast surface tissue formation (in under 3 weeks) In addition, at least one study showed that angiogenesis had occurred. Inert materials will never give such behaviour. The aim of Col-HA composite scaffolds is to enhance the ease of application of tissue engineering, thus such demonstrably efficient bone forming capacity is of great value. Tissue engineered devices will have a direct impact on post-operative recovery times and overall costs of treatment.

Conclusion

This paper has examined the processing routes for fabricating collagen-hydroxyapatite composites and their effect on mechanical and biological properties. It is possible to vary the type of collagen, the crosslinking method and density, the porosity levels and to control the stoichiometry and defect chemistry of the hydroxyapatite, as well as the particle size. The volume fractions of each component are important. Novel techniques such as solid freeform fabrication, coupled with the additions of, for example, growth factors mean that scaffolds made from collagen and hydroxyapatite composites should prove successful in the tissue engineering of hard tissues such as bones.

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Discussion with Reviewers

M.Bohner: One difficulty in producing composite materials for human application is sterilisation. What type of sterilisation could be used in HA-collagen composites?
Authors: An inexpensive sterilisation procedure for collagen-based implants remains to be discovered. Collagen sterilisation ideally should not alter the integrity of the protein's triple helix and/or retain any toxic residues in the process. At the moment, gamma-radiation and ethylene oxide are the most common collagen sterilisation methods used in medical applications but have drawbacks (see "*Note for guidance on limitations to the use of ethylene oxide in the manufacture of medicinal products*" in The European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Human Use, 2001). Experimental lab procedures have sterilised collagen-HA implants using dehydrothermal treatments at 110°C for 2 days or sterile filtered ethanol without inducing protein denaturation, loss in mechanical properties or producing an adverse cellular response.

M.Bohner: The European market is a difficult market for animal- or human-based bone substitutes. Therefore, it would make sense to work with recombinant products. Is it possible to find collagen produced with recombinant technologies, and if yes, how do the mechanical properties of these collagen fibres compare to those retrieved from animals or humans?
Authors: Collagen type I, II and III are examples of proteins that have been produced by recombinant technologies in bacteria (*E.Coli*) and yeast (Pichia pastoris). *An example of an available human recombinant Collagen Type I can be found at www.fibrogen.com. Research on the mechanical properties of human recombinant collagen in comparison to bovine collagen is under investigation currently in our labs. The results will be reported in due course.*

J de Bruijn: When discussing the results of table 4, the authors state that using Col-HA composite scaffolds "at least one study showed that angiogenesis had occurred. Inert materials will never give such behaviour." Please elaborate on this statement and discuss what is so special about Col-HA composite scaffolds to give this behaviour.
Authors: It is important for the biomedical implants to form a direct adhesion with the surrounding cells without any fibrous tissue interface. In bone replacements, type I collagen-based implants have a natural advantage over inert implants by having osteoblast adhesion properties. They contain a specific amino-acid sequence (Arg-Gly-Asp), which can be directly recognised by the cell membrane receptors (e.g. integrins). Inert implants, such as Titanium, require an initial protein adsorption step on its surface before osteoblast adhesion. Endothelial cells (blood vessels cells) can also directly bind to collagen type I. Most importantly, inert materials will not resorb and also cannot be replaced by new tissue.