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Three-dimensional plotted hydroxyapatite scaffolds with predefined architecture: comparison of stabilization by alginate cross-linking versus sintering

Alok Kumar^{1,*}, Ashwini R Akkineni^{2,*}, Bikramjit Basu^{1,3} and Michael Gelinsky²

Abstract

Scaffolds for bone tissue engineering are essentially characterized by porous three-dimensional structures with interconnected pores to facilitate the exchange of nutrients and removal of waste products from cells, thereby promoting cell proliferation in such engineered scaffolds. Although hydroxyapatite is widely being considered for bone tissue engineering applications due to its occurrence in the natural extracellular matrix of this tissue, limited reports are available on additive manufacturing of hydroxyapatite-based materials. In this perspective, hydroxyapatite-based three-dimensional porous scaffolds with two different binders (maltodextrin and sodium alginate) were fabricated using the extrusion method of three-dimensional plotting and the results were compared in reference to the structural properties of scaffolds processed via chemical stabilization and sintering routes, respectively. With the optimal processing conditions regarding to pH and viscosity of binder-loaded hydroxyapatite pastes, scaffolds with parallelepiped porous architecture having up to 74% porosity were fabricated. Interestingly, sintering of the as-plotted hydroxyapatite–sodium alginate (cross-linked with CaCl_2 solution) scaffolds led to the formation of chlorapatite ($\text{Ca}_{9.54}\text{P}_{5.98}\text{O}_{23.8}\text{Cl}_{1.60}(\text{OH})_{2.74}$). Both the sintered scaffolds displayed progressive deformation and delayed fracture under compressive loading, with hydroxyapatite–alginate scaffolds exhibiting a higher compressive strength (9.5 ± 0.5 MPa) than hydroxyapatite–maltodextrin scaffolds (7.0 ± 0.6 MPa). The difference in properties is explained in terms of the phase assemblage and microstructure.

Keywords

Three-dimensional plotting, direct plotting, robocasting, additive manufacturing, rapid prototyping, hydroxyapatite

Introduction

Healthy bone provides the structural stability as well as maintains the Ca^{2+} homeostasis in the body. In natural bone, hydroxyapatite (HA) nanocrystals are embedded in type I collagenous matrix in a hierarchically organized manner and thus the natural bone exhibits anisotropic structure and mechanical properties.¹ HA makes up around 60 wt.% of total inorganic composition of (dry) bone and is fully integrated in the bone remodelling processes.² Although many biocompatible materials have been developed so far for treatment of bone defects, synthetic HA is reported to be most suitable due to its similarity to bone mineral and excellent bioactivity.^{2,3} However, poor mechanical properties (e.g. fracture toughness) of conventionally fabricated HA bioceramics limit its application in load-bearing sites.⁴ In contrast,

spark plasma sintering can be used to fabricate fully dense and crystalline HA with fairly good fracture toughness ($\sim 3 \text{ MPa.m}^{1/2}$) to overcome the brittleness

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aspect to some extent.⁵ Such constructs/implants are however not considered to be suitable for tissue engineering applications due to the absence of open pores, required for tissue ingrowth, vascularization and remodelling.⁶ The crystallinity of HA defines its stability in body fluids at physiological pH. Low crystalline HA with porous architecture is recommended for faster bone regeneration.^{7,8} Superior control over the architecture of the implants can be achieved by various additive manufacturing (AM) techniques such as three-dimensional (3D) printing, 3D plotting or robocasting. Based on the specific fabrication technique used, the structural characteristics such as porosity, pore size and so on can be altered according to the requirement.⁹

Among the manifold AM techniques, adapted nowadays for biomedical applications, 3D powder printing and 3D plotting were found to be most suitable for the processing of calcium phosphate-based materials.^{10,11} By sintering as a post-processing step the mechanical properties (e.g. compressive strength) can be significantly enhanced. In 3D powder printing, the choice of binder is limited as selection of binder depends on the powder composition.^{12,13} Additionally, multiple layers of different composition cannot be printed using commercially available 3D powder printing technology up to now.

In contrast, 3D plotting is a more versatile method than 3D powder printing due to the ease of selection of the (bio)materials to be processed and possibility to plot multiple layers with different composition.^{14–16} 3D plotting is based on the extrusion of pasty materials in a layer-by-layer fashion and has great similarities to well-known fused deposition modelling (FDM) in which polymer melts are extruded. In contrast to FDM, 3D plotting is commonly performed at room or physiological temperature and therefore suitable for processing of thermo-sensitive substances like biopolymers, proteins (growth factors) or even live cells.¹⁷ Additionally, scaffolds with a gradient or layered composition and with defined structure can easily be fabricated using 3D plotting technique.¹⁴ To the best of our knowledge only two studies have been published so far by Lode *et al.*¹⁰ and Akkineni *et al.*,¹⁸ describing the fabrication of a mechanically stable, pure HA scaffold by 3D plotting of a pasty calcium phosphate cement in which all process steps were performed at room temperature. Here, the solid cement precursor particles are suspended in an oily liquid which allows extrusion but does not prevent the setting reaction (i.e. formation of nanocrystalline HA). The self-setting ability of such cement under physiological conditions shows the advantage over other methods which require thermal treatment such as sintering to improve the strength of the scaffolds. The fabricated scaffolds were characterized by 53% of total porosity and compressive strength

of up to ~11 MPa. Importantly, such room temperature processing allows the incorporation of sensitive biological components such as growth factors¹⁸ during printing.

Another AM method, introduced as ‘robocasting’ in the literature, has been demonstrated to be suitable for manufacturing of porous ceramic scaffolds. Robocasting combines the extrusion of a pasty material at room temperature (similar to 3D plotting) with a post-processing sintering step (similar to powder printing). The method was first described by Miranda *et al.* in 2006, using a β -tricalcium phosphate (β -TCP) slurry as paste.¹⁹ The first studies on robocasting of HA were published 1 year later.^{20,21} Dellinger *et al.*²⁰ fabricated such scaffolds with predefined architecture, characterized by macropores of 100–600 μm . Recently, Houmar *et al.*²² fabricated improved scaffolds of HA and HA- β -TCP composites using the robocasting method.

One of the HA-based pastes applied in robocasting was prepared by Saiz *et al.*²¹ by suspending HA powder in water with Darvan® as dispersant. Furthermore, hydroxypropyl methylcellulose and polyethylenimine were added to gellify the suspension, followed by pH adjustment to regulate the viscosity. The plotted scaffolds were sintered at various temperatures ranging between 1100°C and 1200°C to improve the mechanical properties.

Scaffold fabrication by robocasting generally requires a high solid content in a polymeric matrix (to allow extrusion) with desired viscosity (associated with the needle diameter), controlled drying rate (to avoid distortion in shape due to drying) and plottability at room temperature (or below) to manufacture a porous architecture with stable dimensions. Although different HA-based pastes, suitable for 3D plotting, were prepared in the past, the usage of several additives to control the various parameters (e.g. viscosity, injectability, gelification, etc.) makes the ink preparation a very challenging job. Also, such additives may deteriorate the cytocompatibility of non-sintered samples (due to progressive dissolution/degradation of the additives) as well as mechanical properties of sintered samples (due to removal of these additives at high temperature treatment and possible activation of unwanted sintering or side reactions).

In the above perspective, this article will demonstrate the efficacy of 3D plotting as a viable AM method for porous HA scaffolds with aqueous maltodextrin (MA)/polyvinyl alcohol (PVA) mixture or alginate solution (Alg) acting as a binder/carrier material for the mineral phase. The microstructure and mechanical properties are characterized and correlated with reference to phase assemblage, strand and pore characteristics. Finally, the effects of two completely different methods for post-plotting stabilization of the 3D

scaffolds – gelation of Alg with calcium ions versus sintering of the material – are compared.

Materials and methods

Scaffold processing

HA powder (Merck, Germany) with needle-shaped particles (length: 50 ± 4 nm, width: 17 ± 2 nm) was used as a starting material. The particle size and morphology were confirmed by transmission electron microscopy (TEM; F30, FEI, Switzerland, operated at 200 kV). A typical TEM image of the HA powder is provided in Figure 1. For HA–Alg scaffolds, HA powder was mixed in various ratios with 3 wt.% sodium alginate (Alg) (Sigma, Germany) solution in deionized water and 1 M NaOH in order to achieve an optimum viscosity for the 3D plotting process. In the case of HA–MA–PVA scaffolds, 1 g HA was mixed with 0.75 ml maltodextrin (MA) (Tianjiao, China) solution (20 wt.% in water), and 0.5 ml poly(vinyl alcohol) (PVA) (Sigma, Germany) solution (12.5 wt.% in water) to make the paste for plotting. Importantly, no pH adjustment was required in this case and the viscosity was controlled through the adjustment of MA and PVA content.

Both HA–MA–PVA and HA–Alg pastes were characterized for their viscosity using a rotary rheometer (Rheotest® RN 4.1, Germany). A cone/plate with an angle of 1° was used to measure the viscosity of both the paste compositions at dynamic shear rate (increment -0.067 s^{-1}) and at a constant shear rate (20 s^{-1}).

Prior to plotting, all components were mixed gently to prepare a homogenous paste. Scaffold fabrication by 3D plotting was performed with a BioScaffolder 2.1 instrument (GeSiM, Germany) by paste extrusion at room temperature in air. The particular paste was

filled in the syringe with a stopper at the end and centrifuged for 10 min at 2000 rpm to remove trapped air bubbles. The polymer/mineral pastes were used to fabricate 3D scaffolds with dimension of $10 \times 10 \times 7 \text{ mm}^3$ using a needle with an inner diameter of $410 \mu\text{m}$ (Figures 2 and 3) with 5.96 bar air pressure. The plotted scaffolds were cross-linked for 10 h with a 1 M CaCl_2 solution. The cross-linked scaffolds were kept at -20°C for 12 h, followed by freeze drying (Alpha 1-2; Christ, Osterode am Harz, Germany) for 4–6 h. A batch of the dried samples were heated at 450°C for 30 min and then sintered at 1250°C for 1 h in air using conventional pressureless sintering. A muffle furnace (model: L5/13/P330; Nabertherm, Germany) was used for the sintering. Another batch of these scaffolds was characterized directly after freeze drying.

The HA–MA–PVA scaffolds of similar dimensions were also plotted, followed by freeze drying and sintering. These scaffolds were compared with both sintered and non-sintered HA–Alg samples.

Phase assemblage and microstructural characterization

Cross-linked as well as sintered samples were characterized for the phase assemblage using X-ray diffraction (XRD, X'Pert Pro; PANalytical, The Netherlands) with Cu K_α radiation ($\lambda = 0.15056 \text{ nm}$). XRD machine was operated at 40 kV and 30 mA and spectra were acquired with 2θ varying from 20° to 90° , step size of 0.03° and incident angle of 5° .

Scanning electron microscopy (SEM; Inspect F50, FEI, USA), operated in secondary electron mode at an accelerating voltage of 15 kV, was used to investigate the detailed microstructural properties before and after sintering. Prior to this, the dried samples were gold

Figure 1. (a) Representative TEM image of the HA starting material, confirming the needle-shaped particles of 50 ± 4 nm length and 17 ± 2 nm width with aspect ratio of 3. (b) Corresponding selected area diffraction (SAD) pattern confirms the presence of HA phase.

Figure 2. Schematic, showing the scaffold architecture. The scaffold can be considered as a combination of parallelepiped, fabricated layer-by-layer to form the porous 3D structure.

Figure 3. Plot of evolution of torque, viscosity and shear rate with time for HA-MA-PVA (a,c) and HA-Alginate (b,d) pastes under two different measurement conditions: constant shear rate (a, b) and constant increment in shear rate of 0.067 s^{-1} (c, d).

coated using a DESK V magnetron sputter coater (Denton Vacuum, NJ, USA).

A helium gas pycnometer (Ultracyc 1200e, Quantachrome, Germany) was used to measure total

porosity in the freeze dried and sintered scaffolds. Additionally, high resolution micro-computed tomography (micro-CT; Xrdia VersaXRM-500, Zeiss, Germany) was carried out to investigate the

architectural details of the scaffolds, for example, the distribution of pores and pore inter-connectivity. Furthermore, commercially available software 'Avizo Fire' (FEI, USA) was used to estimate the total porosity in the 3D scaffolds. Briefly, for total porosity measurement, a non-local means filter was applied on a slice of 3D reconstructed image, followed by setting of threshold to visualize the pores. After this, volume fraction rendering was applied to calculate the total porosity in the 3D scaffolds.

Mechanical properties

The deformation behaviour of the 3D plotted scaffolds (both freeze dried and sintered samples) was studied under quasi-static compression loading with a pre-load of 1 N, crosshead speed of 5 mm min^{-1} and with upper force limit of 3 kN using an universal testing machine (Z010; Zwick/Roell, Germany, equipped with a 10 kN load cell). Samples with a size of ca. $7 \times 7 \times 5.5 \text{ mm}$ (sintered samples) or ca. $10.8 \times 10.8 \times 7.8 \text{ mm}$ (HA-Alg, non-sintered) were used. A minimum of 6 samples per scaffold type was measured.

Scaffold stability in aqueous conditions

The as-plotted, cross-linked (HA-MA-PVA thermally at 150°C and HA-Alg chemically using 1 M CaCl_2 solution at room temperature) and sintered scaffolds were investigated for their stability in aqueous solution with two different pH values. Distilled water was adjusted to pH of 6 and 7.6 by addition of 1 M HCl and 1 M NaOH solutions, respectively. The scaffolds were then incubated in these solutions at 37°C for 15 h to observe any disintegration.

Results

Plottable paste properties and optimization of processing conditions

As a first step towards scaffold processing, the properties of the plot pastes were optimized in terms of viscosity and injectability. After several trial experiments, 1.25 ml sodium alginate and $50 \mu\text{l}$ of 1 M NaOH solutions were thoroughly mixed with 1 g of HA powder and such paste composition was found to be optimum for plotting at 22°C (room temperature). It has been found that the addition of NaOH lowered the viscosity of the paste. This allows the increase in plotting speed at the cost of inhomogeneity due to less deposition of plotting material. The plotted scaffolds of HA-Alg were cross-linked with 1 M CaCl_2 for structural rigidity and improvement in the green body strength.

While processing HA-MA based scaffold, PVA was added to reduce the viscosity and it was intended that PVA should provide initial strength after a thermal cross-linking at 150°C .²³ However, both freeze dried as well as thermally cross-linked HA-MA-PVA scaffolds get dissolved in both acidic (pH 6) as well as in alkaline solutions (pH 7.6). As expected, no dissolution and therefore no weight loss were recorded in case of sintered samples of both the compositions under above-mentioned conditions (data not shown). Thermally cross-linked HA-MA-PVA scaffolds had to be excluded from further exploration.

In our attempts to understand the viscosity-dependent flowability properties of the HA-MA-PVA and HA-Alg pastes, we have measured the viscosity changes with time at room temperature under two defined conditions – constant shear rate and dynamic shear rate. It is known that under rotary condition, the viscosity is inversely proportional to the rate of shear force applied. As shown in Figure 4(a) and (b), both the pastes exhibit a modest increase in viscosity with time under constant shear rate. However, the viscosity of HA-Alg ($\sim 151 \text{ Pa s}$) is at least three times larger than for HA-MA-PVA paste ($\sim 49 \text{ Pa s}$). Also, the increase in viscosity for HA-MA-PVA paste does not follow a steady increase, at least in the initial period of measurement (up to 150 s). Another point to be noted is that the increment in the viscosity was found to be $\sim 30\%$ and $\sim 26\%$ for HA-MA-PVA and HA-Alg, respectively, under constant shear rate conditions. In contrast, the viscosity of both the pastes sharply decreases, when measured under increasing shear rate and the trend is qualitatively similar (Figure 4(c) and (d)). Interestingly, the final steady-state viscosity of HA-Alg ($\sim 145 \text{ Pa s}$) is once again at least three times larger than in case of HA-MA-PVA paste ($\sim 33 \text{ Pa s}$) under dynamic shear rate measurement conditions.

It is important to discuss the process optimization to plot stable 3D constructs of HA-based materials. After control of viscosity, the plotting speed was adjusted, followed by optimization of tear-off speed (speed of the plotting head required to break the strand after completion of strand deposition) and length (the distance required to break the strand after completion of strand deposition).

As far as other plotting parameters are concerned, the tear-off speed and length determine the plotting of extra length of the strand in addition to pre-programmed strand length. In case of viscoelastic solids, the extra length of plotting material needs to be deposited to avoid an incomplete strand in the plotted scaffold. In the present case (for HA-Alg and HA-MA-PVA), the tear-off speed and tear-off length were set to 1 mm s^{-1} and 1 mm, respectively. In case of plotting materials with high viscosity (faster drying

Figure 4. Photograph, showing the 3D plotting process of HA–Alg and HA–MA–PVA scaffolds at room temperature in air.

rate), it has been noticed that the plotting starts with a delay, which further results in formation of shorter strand than the programmed length. In order to ensure the complete structure manufacturing without any discontinuity in the construct, the start break time (time for which pressure is applied before the plot head moves) and end break time (time for which pressure is applied after the plot head has completed a strand) were set to -0.3 s and 0 s, respectively. In this row, cut-off time for the air pressure before strand break was set to 0 s to avoid the over-deposition of plotting material at the end of the strand.

For a fixed needle diameter and air pressure, the strand diameter of a plotted scaffold strongly depends on the plotting speed. In the present work, slow plotting speed (<1 mm s $^{-1}$) resulted in over-deposition of plotting material with no discontinuity in strand length. This reduced the micro-porosity in strands and therefore increased the strength of both green as well as sintered scaffolds. In contrast, higher plotting speeds (>2 mm s $^{-1}$ in HA–MA–PVA and >5 mm s $^{-1}$ in HA–Alg samples) resulted in less deposition of plotting material and reduced strand diameters. Also, a very high plotting speed (25 mm s $^{-1}$) resulted in discontinuous strands at many places. Considering the above issues, the optimum plotting speed of 2 mm s $^{-1}$ was used for HA–MA–PVA, while HA–Alg was plotted at a speed of 4 mm s $^{-1}$ to fabricate defect-free porous scaffolds.

Microstructure and phase assemblage

The phase retention and/or dissociation in 3D-plotted and freeze-dried as well as sintered scaffolds of HA–Alg and HA–MA–PVA were investigated using slow scan XRD. The resulted diffraction patterns of these samples were

compared with the International Centre for Diffraction Data (ICDD) database to find the phases in the samples. The results of XRD analysis are shown in Figure 5. The diffraction patterns corresponding to as-received HA powder confirm the phase purity (ICDD pdf no.: 74-0566), which is reconfirmed by TEM/selected area electron diffraction analysis (only shown for the starting material in Figure 1(b)). Both XRD and TEM results proved the nano-sized weakly crystalline HA phase. In XRD diffraction spectra, broadening of three major characteristic peaks (corresponding to (211), (112) and (300) planes) as well as other peaks of lower intensity confirms the nano-sized morphology as well as weakly crystalline nature of HA particles in HA–Alg scaffold (as-plotted): The cross-linked, freeze-dried HA–Alg samples showed broad XRD patterns, resembling the true features of nano-composite materials, which is obvious due to the presence of alginate. Sintering leads to an increase in crystallinity of inorganic phases as well as removal of organic phase due to heating at 1250°C for 2 h. Importantly, no other phase (e.g. TCP), except HA was found in the sintered HA–MA–PVA samples. It appears that the MA and/or PVA stabilize the HA phase at higher temperature (above 1000°C) and therefore retarded the transformation of HA into TCP. Also, no shift in XRD peaks was noticed, which confirms the absence of residual stress in the sintered samples. The presence of residual stress results in the time-dependent deformation in the sample, which is not desirable for the materials for structural applications. Surprisingly, no peak corresponding to HA was found in case of sintered HA–Alg samples. Also, no peaks corresponding to β -TCP was found, which confirms that HA has not dehydroxylated into TCP phase, which is very usual in HA-based system, when heated at a temperature higher than 1000°C.

Figure 5. (a) Heating cycle for the sintering of HA–MA–PVA and HA–Alg scaffolds. (b) Sintered HA–MA–PVA scaffold, showing no visible deformation in structure due to sintering and the presence of well-defined macropores. (c–e) Micro-CT images of sintered scaffold (HA–MA–PVA) confirm the interconnected porous structure with predefined pore architecture.

After careful analysis it was found that HA ($\text{Ca}_{10}\text{P}_6\text{O}_{24}(\text{OH})_2$) had been transformed into chlorapatite ($\text{Ca}_{9.54}\text{P}_{5.98}\text{O}_{23.58}\text{Cl}_{1.60}(\text{OH})_{2.74}$; ICDD pdf no.: 70-0795) in the presence of NaCl. The three major characteristic peaks of chlorapatite appear at 2θ values of 31.47° , 32.18° and 32.48° , which correspond to (211), (112) and (300) planes, respectively. It is plausible that NaCl crystal is formed as a side-product of cross-linking of sodium alginate with CaCl_2 .

Since the sintering resulted in uniform shrinkage of $\sim 30\%$ (in dimension) in both the compositions, therefore, required adjustment of scaffold dimensions was considered prior to the plotting. Importantly, shrinkage did not lead to structural deformation and thus the architecture of pores was preserved during sintering. The sintering also resulted in grain growth with average grain size of $\sim 870\text{ nm}$ (Figure 6), which is 17 times larger than that of the starting HA particles ($\sim 50\text{ nm}$ length and $\sim 17\text{ nm}$ width; Figure 1).

Careful SEM observation of the scaffolds revealed the presence of cracks on the strands of sintered HA–MA–PVA scaffolds (marked with white arrow, Figure 7). No such cracks were found in freeze-dried as well as sintered HA–Alg scaffolds. However, the

Figure 6. Representative XRD spectra, showing the presence of HA ($\text{Ca}_{10}\text{P}_6\text{O}_{24}(\text{OH})_2$) phase in HA–Alg (cross-linked and freeze-dried) and sintered HA–MA–PVA scaffolds. The transformation of HA into chlorapatite ($\text{Ca}_{9.54}\text{P}_{5.98}\text{O}_{23.58}\text{Cl}_{1.60}(\text{OH})_{2.74}$) was found in sintered HA–Alg samples.

presence of micro-pores was noticed in the sintered microstructure of both HA-MA-PVA as well as HA-Alg scaffolds.

The micro-CT analysis of the scaffolds confirmed the presence of interconnected macro-pores and uniform strand thickness. As shown in Figure 7, the plotted scaffolds are characterized by relatively small pores between two consecutive strands in Z-direction ($\sim 83\mu\text{m}$ in case of sintered HA-MA-PVA scaffolds and $\sim 171\mu\text{m}$ in case of sintered HA-Alg scaffolds) compared to X- and Y-direction (direction of loading in compressive strength measurement; here $\sim 500\mu\text{m}$ for both types of sintered scaffolds). Such open porous structure is highly appreciated for bone tissue engineering applications. No structural deformation in non-supported areas (i.e. strand segments above a pore) was found, although additional material acting as porogen was not provided.

Compression properties

Figure 8(a) shows the porosity and compressive strength of freeze-dried HA-Alg as well as sintered HA-Alg and HA-MA-PVA samples. From this graph it is evident that the compressive strength strongly depends on porosity as well as scaffold composition. A decrease in total porosity from $\sim 74\%$ to $\sim 44\%$ results in an increase in compressive strength from ~ 2.6 to ~ 9.5 MPa, respectively. However, a further decrease in compressive strength from ~ 9.5 to ~ 7 MPa without any change in porosity can be correlated with the phases present in the samples as well as grain size. Importantly, the major phase in sintered HA-Alg samples was chlorapatite in contrast to the predominant presence of HA in sintered HA-MA-PVA samples. A modest improvement in the compressive strength therefore can be attributed to the presence of chlorapatite, when compared with HA (Table 1).

Figure 7. Representative SEM images of cross-linked HA-Alg scaffold after freeze-drying and sintered HA-Alg and HA-MA-PVA scaffolds. High magnification images show the fully densified microstructure with nanosized grains in case of the sintered samples and preservation of the nanocrystalline HA in the non-sintered material. The presence of NaCl crystals can be noticed, formed due to reaction of Na-alginate with CaCl_2 .

Figure 8. (a) Representative line plots, showing variation in ultimate compressive strength with respect to total porosity as well as stabilization regime, applied to the scaffolds. A minimum of six samples were measured per type of scaffold. (b) Representative stress–strain curves generated during unidirectional compressive loading of HA–Alg porous scaffolds (sintered vs. non-sintered).

Figure 8(b) represents the synergistic effect of strands in multi-layered 3D scaffolds during progressive failure after reaching the ultimate compressive strength. In case of sintered samples, the role of strands in mechanical stability of the scaffolds is clearly visible. In contrast, the strain–stress curve of cross-linked only (non-sintered) samples of the same composition in compressive loading shows the characteristic behaviour similar to that of thermoplastic polymers. The slope of the initial linear part of the stress–strain curves (compressive modulus) for sintered and cross-linked only HA–Alg scaffolds were 1.69 and 0.48 MPa, respectively. Apart from higher compression modulus, the strain to failure for sintered scaffolds is 2.6–2.7% compared to that for cross-linked only HA–Alg scaffolds (~21–22%). Interestingly, the cross-linked HA–Alg scaffolds showed large strain at constant stress (~2 MPa).

Discussion

The present work highlights the preparation of HA-based plottable pastes (HA–MA–PVA and HA–Alg) and also presents the results of fabrication of HA-based open-porous 3D scaffolds using 3D plotting. A short-term (12 h) dissolution test, carried out at room temperature in both slightly acidic as well as alkaline media, showed fast dissolution (data not shown) of both only freeze-dried as well as thermally cross-linked HA–MA–PVA scaffolds. Therefore, in this study, only freeze-dried and cross-linked (in CaCl_2 solution) samples of HA–Alg were compared with sintered samples of HA–MA–PVA and HA–Alg compositions.

Comparison between chemical versus sintering-induced stabilization

During sintering, the intermediate holding at 450°C for 30 min leads to complete removal of polymeric phase and thus resulted in pure inorganic, micro-porous 3D structures. Further heating at 1250°C for 1 h leads to densification and thus reduction in micro-porosity. One can see the presence of only few open micro-pores in the sintered samples, both HA–MA–PVA and HA–Alg (Figure 7), although the presence of closed (internal) porosity cannot be excluded in SEM observations.

The starting powder was characterized as nano-crystalline HA. In freeze-dried scaffolds as well as sintered HA–MA–PVA samples, no other phase except HA was detected. In contrast, the sintering of HA–Alg samples at 1250°C for 1 h leads to the complete transformation of HA to chlorapatite. The reactions associated with this transformation can be written as follows:

Although XRD analysis clearly claims the formation of chlorapatite ($\text{Ca}_{9.54}\text{P}_{5.98}\text{O}_{23.8}\text{Cl}_{1.60}(\text{OH})_{2.74}$) along with CaO in reaction 2, the sintering at 1250°C also increases crystallinity of HA. Importantly, highly

Table 1. Comparison of the results of the present work with selected published studies on 3D plotting of bioceramic and composite scaffolds.

No.	Material	Total solid content in carrier liquid	Solidification method	Average spacing between the layers in direction of loading (μm)	Average strand diameter (μm)	Unit cell of 3D scaffold	Average total porosity (%)	Grain size in sintered scaffold (μm)	Compressive strength (MPa)	Reference
1.	HA-Darvan ® C-hydroxypropyl methylcellulose-polyethylenimine	35 (vol%)	Printed scaffolds were dried in air and then at 400°C, followed by sintering at 1300°C	300 $\pm$ 10	220 $\pm$ 10		39	3.2 $\pm$ 0.5	~45	24
2.	β -TCP-Darvan ® C-hydroxypropyl methylcellulose-polyethylenimine	45 (vol%)	Dried in air and then at 400°C, followed by sintering at 1300°C	300 $\pm$ 10	220 $\pm$ 10		40	7.4 $\pm$ 0.7	~12	25
3.	HA-Pluronic ® solution-corn syrup	47 (vol%)	Dried at room temperature and then at 600°C, followed by sintering at 1275°C	40	~80		—	~6	—	36
4.	α -TCP-gelatin (transformed to HA-gelatin in water)	1.5 (g ml ⁻¹)	Printed scaffolds immersed in a mixture of EDC carbodiimide and NHS for gelatin cross-linking, followed by drying at room temperature	170 $\pm$ 40	~850		60	—	16.6 $\pm$ 3.2	27
5.	HA-forming calcium phosphate cement ^a with a solution of triglyceride, Tween 80 and phosphoric acid monohexadecyl ester	4 (g/g)	Printed scaffolds were immersed in humidity for 3 days for cement setting, following by drying	—	680		53	—	11.4 $\pm$ 4.6	18
6.	HA suspension (in deionized water) dispersed in ammonium polyacrylate.	48 (vol%)	Air drying at room temperature for 1 h, followed by sintering for 2 h at 1200°C	268	539		—	—	—	20

(continued)

Table I. Continued

No.	Material	Total solid content in carrier liquid	Solidification method	Average spacing between the layers in direction of loading (μm)	Average strand diameter (μm)	Unit cell of 3D scaffold	Average total porosity (%)	Grain size in sintered scaffold (μm)	Compressive strength (MPa)	Reference
	Hydroxypropyl methycellulose as a viscosifier		Printed scaffolds were air dried at room temperature, followed by sintering at 1300°C	276	537		—	—	—	
7.	HA mixed with aqueous pluronic F-127 solution	2.8 (wt./wt.)	Printed scaffolds were air dried at room temperature, then heated at 600°C to remove the organic binder, followed by sintering at 1275°C	—	—		25 60	—	~50 ~7.5	22
8.	Mixed CaP cement ^a -Alg-PVA (not sintered)	—	Cross-linked with CaCl_2 , and then incubated in deionized water, followed by drying at room temperature	422 ± 42	700 ± 15		70.8 ± 6.6	—	~5.8	14
9.	HA-MA-PVA	0.8 g ml^{-1} (MA-PVA)	Freeze dried and then sintered at 1250°C	82 ± 9.68	350 ± 15		44 ± 1.7	0.97	7 ± 0.6	Present work
10.	HA-Alg (non-sintered)	0.8 g ml^{-1} (Alg-NaOH)	Cross-linked with CaCl_2 , followed by freeze drying	~10	510 ± 24		74.3 ± 0.3	~0.034	2.6 ± 0.1	Present work
11.	HA-Alg	0.8 g ml^{-1} (Alg-NaOH)	Cross-linked with CaCl_2 , followed by freeze drying and then sintered at 1250°C	170 ± 7.56	426 ± 10		44 ± 3	0.77	9.5 ± 0.5	Present work

EDC: 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide; NHS: N-hydroxysuccinimide.

^a60 wt.% α -TCP, 26 wt.% dicalcium phosphate anhydride, 10 wt.% calcium carbonate and 4 wt.% HA.

crystalline HA shows poor dissolution under physiological conditions and can be considered as nearly non-biodegradable.⁷ The uniform shrinkage with no structural deformity can be correlated with the low binder content. Thus, the removal of binder does not lead to structural deformation. Also, the effect of CaCl_2 (cross-linker for sodium alginate) on the shrinkage of HA–Alg was noticed, where CaCl_2 may promote the transformation to chlorapatite. The high mineral content ensures the particle-to-particle contact during sintering and results in a rather densified microstructure (Figure 7). However, the presence of few micro-pores in the sintered microstructure can be correlated with the removal of polymer during heating. Few cracks were also found on the strands of sintered HA–MA–PVA scaffolds. However, no such crack was noticed in the freeze-dried samples of either composition. Therefore, these cracks must have been generated during the cooling cycle of sintering. Notably, microstructure of the sintered scaffolds of HA–MA–PVA and HA–Alg are characterized by sub-micron grains with average grain size of 970 (HA–MA–PVA) and 770 nm (MA–Alg). It has been reported that such microstructures enhance osteoblast function in terms of proliferation and alkaline phosphatase expression.²⁸

The sintering resulted in a 30% decrease in total porosity (from 74% to 44%) due to associated overall shrinkage. The porosity is bound to affect the mechanical properties significantly and a strong effect of sintering on the compressive strength could be observed in the present case (Figure 8). Sintering results in the consolidation of loosely bound particles, which improves the mechanical properties of the scaffold.²⁹ Therefore, in case of sintered scaffolds, a higher value of compressive strength (~ 9.5 MPa for HA–Alg and ~ 7 MPa for HA–MA–PVA) was measured. The lower value of compressive strength for HA–MA–PVA scaffolds can be correlated with the presence of micro-cracks on the strand surface (Figure 7). The scaffold architecture also plays an important role in strength and stability under compressive loading. After the failure of the first layer of the scaffold, load is transferred to the subsequent unbroken layers and thus prevents an immediate catastrophic failure of the scaffold under loading (Figure 8(b)). Such failure mechanism is not usual in bulk monolithic HA samples, where material fails in an uninterrupted manner under loading in the absence of crack inhibition mechanisms.

Comparison with literature results

Finally, a comparison of the present results with those reported in literature is given in Table 1, wherein 3D plotting (or similar, extrusion-based AM methods) was used to fabricate calcium phosphate bioceramic

and selected calcium phosphate/polymer composite scaffolds.

Concerning the post-fabrication stabilization regime we have to distinguish between three different methods: First, sintering; second, chemical cross-linking of organic components of the plotting paste and third, utilization of self-setting calcium phosphate cement pastes. In case of HA and other calcium phosphate phases, the sintering at temperatures above 1000°C leads to complete removal of all non-mineral components and formation of a mostly dense and mechanically stable microstructure. Macro-pores remain unaffected but decrease in size due to the general shrinkage during debinding, whereas thermal debinding leaves behind a micro-porous surface which can improve cell adhesion due to the presence of mechanical anchoring sites and thus enhances the cell-material interaction.³⁰ It is worthwhile to note that sintering is accompanied by grain growth of the respective mineral phase, leading to mechanically more reliable materials, but such ceramics (if consisting of pure HA) cannot be degraded by osteoclasts anymore. In addition, the high temperature treatment prevents any functionalization of the material with other compounds, like drugs or growth factors during scaffold fabrication.

The stabilization of plotted scaffolds by chemical cross-linking leads to composite materials consisting of the (mostly organic) pasty matrix and the calcium phosphate mineral phase. Many studies on fabrication of such composites by AM technologies and their properties have been published and therefore were not enclosed in Table 1 with the exception of the material (HA–Alg, non-sintered) described in this study. Due to the presence of the elastic organic matrix in the final scaffold material, the mechanical properties are very different to those of the sintered, pure calcium phosphate bioceramics. The composites are less brittle, but are also less strong. Depending on the cross-linking reaction, sensitive components like growth factors or even live cells can be integrated already during the plotting process. This is possible for non-covalent cross-linking (like gelation of alginate in the presence of calcium ions), but not for covalent cross-linking (e.g. amide formation using 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide as chemical cross-linker).

Finally, a few studies on self-setting calcium phosphate cements and porous scaffolds made thereof by 3D plotting were published so far. Here, the final product (after cement setting) can be a pure calcium phosphate phase like in the case of the sintered samples – but without the need of a high temperature consolidation step. In case of HA-forming cements, the setting can be achieved at neutral pH and room temperature, again allowing incorporation of sensitive additional components. Recently, Akkineni *et al.*¹⁸ could

demonstrate that the vascular endothelial growth factor can be incorporated in such a HA-forming cement prior to scaffold fabrication by 3D plotting – and is released in biologically active form after cement setting.

A direct comparison of the material properties of single types of scaffolds is difficult not only because of the different stabilization regimes, but also due to the very diverse geometry and porosity of the scaffolds, which have been fabricated by other groups. Especially mechanical properties are known to be more influenced by total porosity than by small differences in material composition. In the following, some of the material properties, listed in Table 1, will be highlighted. As described before, mechanically most stable scaffolds can be achieved by sintering. The highest compressive strength value published so far for 3D plotted HA scaffolds is around 50 MPa for a scaffold with only 25% total porosity,²² followed by 45 MPa, measured for a material with 39% total porosity.²⁴ The two novel compositions described in this study have a compressive strength of 7 (HA–MA–PVA) and 9 MPa (HA–Alg), respectively, both with a total porosity of 44%. In contrast, the non-sintered HA–Alg scaffolds, which were stabilized by non-covalent alginate cross-linking, exhibit only a compressive strength of 2.6 MPa – but have a very high total porosity of 74% in dry state. Nevertheless, this type of scaffold is stable enough for handling under cell culture conditions and therefore can be used as matrix for tissue engineering applications.

Only very few studies on 3D plotting of HA-forming, self-setting calcium phosphate cements were published so far. Lode *et al.*¹⁰ described such a material having a compressive strength of 6.1 MPa at 56% total porosity. Very recently, the same group demonstrated that mechanical strength can be improved by conducting the setting reaction in saturated humidity instead of immersion in water. By applying this novel setting regime, 3D plotted scaffolds with a total porosity of 53% were demonstrated to exhibit a compressive strength of 11.4 MPa.¹⁸ A similar approach was used by another study, but combined with chemical (covalent) cross-linking of a gelatin hydrogel. This combined stabilization strategy (cement setting and covalent biopolymer cross-linking) led to a final strength of 16.6 MPa for scaffolds with 60% total porosity.²⁷ Unfortunately, this cross-linking regime prevents the incorporation of delicate substances like drugs or growth factors. Due to the absence of any thermal treatment, the product of the setting reactions in all these cases is nano-crystalline HA, which is known to be fully degradable by osteoclasts *in vivo*.

Conclusions

The present work demonstrates the usefulness of the extrusion-based AM technology of 3D plotting for

fabrication of clinically relevant 3D scaffolds of HA–MA–PVA and HA–Alg with pre-defined porosity. These scaffolds can be manufactured with a large-scale macro-porosity of ~74% in non-sintered and ~44% in sintered samples. In contrast, HA–MA–PVA remains unstable under aqueous conditions without sintering. Our experimental results also suggest that HA–Alg scaffolds either can be stabilized by non-covalent cross-linking of alginate with calcium ions or by sintering. Such porous scaffolds with a large volume of pre-defined porosity can be used as substrate for tissue engineering or as bone defect filler due to interconnected porous network. Interestingly, the presence of chlorapatite ($\text{Ca}_{9.54}\text{P}_{5.98}\text{O}_{23.8}\text{Cl}_{1.60}(\text{OH})_{2.74}$) was recorded in sintered HA–Alg scaffolds only.

The layered structure of the scaffolds was found to be helpful in improving the compressive strength due to progressive failure under compressive loading instead of catastrophic or uninterrupted failure, commonly exhibited by sintered bulk ceramics. As compared to sintered HA–MA–PVA scaffolds (7.0 ± 0.6 MPa), a higher compressive strength has been measured in case of sintered HA–Alg scaffolds (9.5 ± 0.5 MPa). In contrast, a lower value of compressive strength (2.6 ± 0.1 MPa) has been measured in non-sintered HA–Alg scaffolds, stabilized by alginate cross-linking. Overall, the strength of the non-sintered as well as sintered scaffolds was found to fall in the range of cancellous bone (2–12 MPa).

The present work also demonstrates the specific advantages and disadvantages of chemical stabilization and sintering-induced stabilization of HA-based scaffolds, while sintering generally leads to higher compressive strength but simultaneously makes the scaffold mostly non-biodegradable. In contrast, chemical stabilization by non-covalent cross-linking of alginate results in mechanically less-stable scaffolds, but enables the composite to be degradable. In addition, an absence of the sintering step opens up the possibility of incorporating sensitive biological components such as drugs or proteins (like growth factors) during the plotting process, which strongly extends the utilization of such materials in tissue engineering.

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References

1. Rho JY, Kuhn-Spearing L and Zioupos P. Mechanical properties and the hierarchical structure of bone. *Med Eng Phys* 1998; 20: 92–102.
2. Murugan R and Ramakrishna S. Development of nanocomposites for bone grafting. *Compos Sci Technol* 2005; 65: 2385–2406.
3. Dorozhkin S. Calcium orthophosphate-based bioceramics. *Materials (Basel)* 2013; 6: 3840–3942.
4. Kumar A, Biswas K and Basu B. Hydroxyapatite-titanium bulk composites for bone tissue engineering applications. *J Biomed Mater Res A* 2014; 103: 791–806.
5. Kumar A, Biswas K and Basu B. On the toughness enhancement in hydroxyapatite-based composites. *Acta Mater* 2013; 61: 5198–5215.
6. Feng B, Jinkang Z, Zhen W, et al. The effect of pore size on tissue ingrowth and neovascularization in porous bioceramics of controlled architecture *in vivo*. *Biomed Mater* 2011; 6: 015007.
7. Fulmer MT, Ison IC, Hankermayer CR, et al. Measurements of the solubilities and dissolution rates of several hydroxyapatites. *Biomaterials* 2002; 23: 751–755.
8. Karageorgiou V and Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials* 2005; 26: 5474–5491.
9. Vasireddi R and Basu B. Conceptual design of three-dimensional scaffolds of powder-based materials for bone tissue engineering applications. *Rapid Prototyp J*. Epub ahead of print 2015. DOI: 10.1108/RPJ-12-2013-0123.
10. Lode A, Meissner K, Luo Y, et al. Fabrication of porous scaffolds by three-dimensional plotting of a pasty calcium phosphate bone cement under mild conditions. *J Tissue Eng Regen Med* 2014; 8: 682–693.
11. Castilho M, Dias M and Gbureck U. Fabrication of computationally designed scaffolds by low temperature 3D printing. *Biofabrication* 2013; 66: 911–917.
12. Klammert U, Gbureck U, Vorndran E, et al. 3D powder printed calcium phosphate implants for reconstruction of cranial and maxillofacial defects. *J Craniomaxillofac Surg* 2010; 38: 565–570.
13. Chumnanklang R, Panyathanmaporn T, Sitthiseripratip K, et al. 3D printing of hydroxyapatite: effect of binder concentration in pre-coated particle on part strength. *Mater Sci Eng C* 2007; 27: 914–921.
14. Luo Y, Lode A, Sonntag F, et al. Well-ordered biphasic calcium phosphate–alginate scaffolds fabricated by multichannel 3D plotting under mild conditions. *J Mater Chem B* 2013; 1: 4088–4098.
15. Landers R, Hübner U, Schmelzeisen R, et al. Rapid prototyping of scaffolds derived from thermoreversible hydrogels and tailored for applications in tissue engineering. *Biomaterials* 2002; 23: 4437–4447.
16. Luo Y, Akkineni AR and Gelinsky M. Three-dimensional plotting is a versatile rapid prototyping method for the customized manufacturing of complex scaffolds and tissue engineering constructs. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2014; 28: 279–285.
17. Bajaj P, Schweller RM, Khademhosseini A, et al. 3D biofabrication strategies for tissue engineering and regenerative medicine. *Annu Rev Biomed Eng* 2014; 16: 247–276.
18. Akkineni AR, Luo Y, Schumacher M, et al. 3D plotting of growth factor loaded calcium phosphate cement scaffolds. *Acta Biomater* 2015; 27: 264–274.
19. Miranda P, Saiz E, Gryn K, et al. Sintering and robocasting of beta-tricalcium phosphate scaffolds for orthopaedic applications. *Acta Biomater* 2006; 2: 457–466.
20. Dellinger JG, Cesarano J and Jamison RD. Robotic deposition of model hydroxyapatite scaffolds with multiple architectures and multiscale porosity for bone tissue engineering. *J Biomed Mater Res A* 2007; 82A: 383–394.
21. Saiz E, Gremillard L, Menendez G, et al. Preparation of porous hydroxyapatite scaffolds. *Mater Sci Eng C* 2007; 27: 546–550.
22. Houmard M, Fu Q, Genet M, et al. On the structural, mechanical, and biodegradation properties of HA/ β -TCP robocast scaffolds. *J Biomed Mater Res B Appl Biomater* 2013; 101: 1233–1242.
23. Wu C, Fan W, Zhou Y, et al. 3D-printing of highly uniform CaSiO₃ ceramic scaffolds: preparation, characterization and *in vivo* osteogenesis. *J Mater Chem* 2012; 22: 12288–12295.
24. Miranda P, Pajares A, Saiz E, et al. Fracture modes under uniaxial compression in hydroxyapatite scaffolds fabricated by robocasting. *J Biomed Mater Res A* 2007; 83: 646–655.
25. Miranda P, Pajares A, Saiz E, et al. Mechanical properties of calcium phosphate scaffolds fabricated by robocasting. *J Biomed Mater Res Part A* 2008; 85: 218–227.
26. Franco J, Hunger P, Launey M, et al. Direct write assembly of calcium phosphate scaffolds using a water-based hydrogel. *Acta Biomater* 2010; 6: 218–228.
27. Maazouz Y, Montufar EB, Guillem-Marti J, et al. Robocasting of biomimetic hydroxyapatite scaffolds using self-setting inks. *J Mater Chem B* 2014; 2: 5378–5386.
28. Webster TJ, Ergun C, Doremus RH, et al. Enhanced functions of osteoblasts on nanophase ceramics. *Biomaterials* 2000; 21: 1803–1810.
29. Kang S-JL. *Sintering: densification, grain growth and microstructure*. Oxford, UK: Elsevier Butterworth-Heinemann, 2004.
30. Chanda A, SinghaRoy R and Xue W. Bone cell materials interaction on alumina ceramics with different grain sizes. *Mater Sci Eng C* 2009; 29: 1201–1206.