Simple additive manufacturing of an osteoconductive ceramic using suspension melt extrusion

Slots, Casper; Bonde Jensen, Martin; Ditzel, Nicholas; Hedegaard, Martin A B; Borg, Søren Wiatr; Albrektsen, Ole; Thygesen, Torben; Kassem, Moustapha; Andersen, Morten Østergaard

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Abstract

Objectives

Craniofacial bone trauma is a leading reason for surgery at most hospitals. Large pieces of destroyed or resected bone are often replaced with non-resorbable and stock implants, and these are associated with a variety of problems. This paper explores the use of a novel fatty acid/calcium phosphate suspension melt for simple additive manufacturing of ceramic tricalcium phosphate implants.
Methods

A wide variety of non-aqueous liquids were tested to determine the formulation of a storable 3D printable tricalcium phosphate suspension ink, and only fatty acid-based inks were found to work. A heated stearic acid-tricalcium phosphate suspension melt was then 3D printed, carbonized and sintered, yielding implants with controllable macroporosities. Their microstructure, compressive strength and chemical purity were analyzed with electron microscopy, mechanical testing and Raman spectroscopy, respectively. Mesenchymal stem cell culture was used to assess their osteoconductivity as defined by collagen deposition, alkaline phosphatase secretion and de-novo mineralization.

Results

After a rapid sintering process, the implants retained their pre-sintering shape with open pores. They possessed clinically relevant mechanical strength and were chemically pure. They supported adhesion of mesenchymal stem cells, and these were able to deposit collagen onto the implants, secrete alkaline phosphatase and further mineralize the ceramic.

Significance

The tricalcium phosphate/fatty acid ink described here and its 3D printing may be sufficiently simple and effective to enable rapid, on-demand and in-hospital fabrication of individualized ceramic implants that allow clinicians to use them for treatment of bone trauma.
Keywords: Ceramic, Bone Implant, Additive Manufacturing, Tricalcium Phosphate, 3D Printing, Implant

1. Introduction

Bone trauma is a common condition that is treated at most hospitals and which may occur due to a number of reasons such as accidents, falls, violence, surgery, infection, degenerative diseases and cancer resection [1],[2]. Some bone defects and fractures are treated with titanium mini-plates and screws as bone heals on its own. However, permanent implants may be required to replace pieces of resected or destroyed bone that are either too large to heal on their own or where the restoration of a particular anatomical feature is critical to the aesthetic or functional outcome [3]. The current paper focuses on this type of implant. Autologous bone may be used for such implants but tissue harvesting results in increased morbidity and increased time of surgery. Implants made from non-resorbable materials are common alternatives, but, depending on the type of defect and type of implant, such materials are at risk of infection and rejection and may stress shield surrounding bone, generate wear debris and loosen over time. Furthermore, non-resorbable implants cannot be repaired and remodeled by the body and often need to be replaced in young growing patients. Degradable (resorbable) implant materials may solve these problems [4]. Ceramic calcium phosphate-based implants are an established alternative to non-resorbable implant materials and autologous bone and have been used clinically for many years [5]. Tricalcium phosphate (TCP) is especially interesting as it slowly biodegrades and is remodeled to true bone to which it contributes calcium and phosphate. To accelerate new bone formation, bone forming cells such as mesenchymal stem cells (MSCs) are often added to implants [6],[7],[8].

To further improve the use of resorbable, tricalcium-based implants, the use of three dimensional (3D) printing has evolved to a level at which such techniques can actually contribute to more predictable reconstructions. 3D printing, a method of additive manufacturing, is a computer-assisted manufacturing method where an object is
built layer-by-layer using a digital object model also known as a CAD model. It has recently been applied to the fabrication of bone implants as it can be used to produce individualized implants that recapitulate patient anatomy especially when used in conjunction with patient scanning data and virtual surgical planning [9,10]. 3D printing also enables the formation of bone-forming pores with specific diameters and direction within the implant. TCP can be 3D printed using different methods, including stereolithography [11], selective laser sintering [12], binder ink jetting [13],[14],[15] and robocasting. Robocasting, also known as direct writing and extrusion printing, is an additive manufacturing technique where an “ink” is deposited by a computer-controlled extruder onto a build platform [16]. Robocasting is an attractive technique for in-hospital 3D printing due to its simplicity, low-cost, low-maintenance and its capability to use any material and combinations of several different materials in one print [17]. Several previous studies have used robocasting to produce calcium phosphate implants that have successfully supported osteogenesis in vitro and in vivo [18],[19],[20],[21],[22]. Recently, a number of simple-to-operate and low-cost (under $ 10,000) robocasting capable 3D printers have become commercially available; these include the Biobot’s Biobot [23], Cellink’s Inkredible [24] and Hyrel’s System 30M [25]. The simplicity and pricing of these 3D printers could enable low-cost and on-demand 3D printing of individualized implants locally at hospitals. This also requires an ink that is equally low-cost and simple to prepare and handle.

Most publications concerning robocasting describe inks that are complex aqueous powder suspensions that contain multiple additives and which often take several hours to prepare. Aqueous suspension may also suffer from problems such as detrimental influence from their pH and uneven dehydration during printing. Strategies for improving robocasting such as using inks that gel independently of pH [26] or by printing into an oil bath [27] are therefore of great interest. However, all water-based prints must be fully dehydrated before sintering as the boiling water produced in the process would otherwise introduce cracks in the final ceramic. This dehydration step typically takes one or more days. Aqueous formulations may also support chemical reactions
such as oxidation, hydration or recrystallization of the powder, resulting in a non usable ink; this precludes extended storage and requires fresh inks to be prepared for each print. The need for fresh inks, the complexity and time required to prepare such inks and the extended post-printing dehydration time present a significant obstacle to rapid, local, on-demand printing of implants in hospitals.

Long aliphatic chains efficiently lubricate surfaces, and molecules that contain such chains are often used as lubricants. Stearic acid, for example, lowers inter-particle attraction and granular shear strength [28]. This paper investigates the use of such lubricants to create a new calcium phosphate implant fabrication method that relies on a simple-to-prepare, two-component, non-aqueous robocasting ink. Our aim was to develop a simple and inexpensive individualized implant production method that could be implemented at hospitals without compromising implant strength, biocompatibility or osteoconductivity.

2. Methods

2.1 Materials

Tricalcium phosphate (TCP, Cat. No. 21218), ascorbic acid, dexamethasone, calcitriol, betaglycerol phosphate and stearic acid, copper powder and copper oxide were purchased from Sigma Aldrich (St. Louis, MO, USA). MEM medium, penicillin/streptomycin (P/S), trypsin and fetal bovine serum (FBS) were acquired from Invitrogen (Waltham, MA, USA).

2.2 Ink Testing
To replace water in the robocasting ink, TCP was combined at various ratios with a large number of potential lubricants including decane, oleic acid, oleyl alcohol, oleyl ester, glycerol, triglycerides (sunflower oil) and heated paraffin. The viscoelastic properties of the inks were tested qualitatively by stirring the suspensions with a spoon. Their extrusion/3D printing properties were tested by placing them in an aluminum syringe that was placed into a System 30 3D printer (Hyrel 3D, Atlanta, GA, USA). Using the printer’s manual settings, the syringe was then pressurized and the extruded liquid or suspension was visually and physically inspected.

2.3 Implant Preparation

Inks for implant preparation were formulated by mixing 25g TCP with 5g stearic acid (W/W 83%, V/V 60%) and heating the mixture to 80°C. This ink was loaded into an aluminum syringe that was placed into a heatable syringe extrusion head (a Vol-25, Hyrel 3D, Atlanta, GA, USA). CAD files of 50 mm x 50 mm x 3 mm or 20 mm x 20 mm x 20 mm boxes were constructed using Autodesk Inventor 2015 and exported as STL files. This files were imported into a System 30 3D printer, where they were prepared for printing using Slic3r 1.2.9. Settings were a layer height of 0.2 mm, a print speed of 15 mm/s, an infill of 70%, a rectangular infill pattern, a solid top and bottom value of 0 and a perimeter value of 0. The extrusion head was heated to 80°C and remained at this temperature during the entire print. The printing bed was heated to 40°C for the first layer of the print to facilitate ink adhesion but was not heated for the remaining prints where the approximate bed temperature was 25 °C. Printing was done through a 1 mm nozzle. After printing, the 50 mm x 50 mm x 3 mm print was carefully removed from the printing bed and was cut into smaller 7 mm x 7 mm x 3 mm implants containing four pores. The smaller implants were not printed directly as the 3D printer software used at the time of the study had difficulty printing the edges of small objects, by printing and trimming larger objects this problem could be eliminated. This is not a problem for clinical implementablity as defects that small would self-heal.
anyway. The larger 20 mm x 20 mm x 20 mm implants, a clinically relevant size, were 3D printed directly. Except where noted, all implants were placed into a pre-heated oven (Nabertherm L3) for 1 h at 400°C and were then sintered for 2 hours at 1100°C followed by slow cooling to room temperature (1–2 hours). All experiments were conducted with the 7 mm x 7 mm x 3 mm implants, except for the mechanical testing.

2.4 Cell Seeding and Differentiation

For the initial experiment three scaffolds were placed in each well on ultra-low-adherence 24-well plates (Corning) that was then added 100,000 cells eGFP$^+$ hMSC (Tert4$^+$, p62) cells in 500 µL maintenance medium (MEM medium with 1% P/S and 10% FBS). For the remaining experiments with eGFP hMSCs, single implants were placed in clean ultra-low-adherence 24-well plates to which was added 200,000 eGFP$^+$ hMSCs (Tert4, p45) in 50 µL maintenance medium. After 30 minutes, 1 mL medium was added to each well. After 48 hours, the medium was replaced with either 1 mL maintenance medium or osteogenic medium (maintenance medium plus 10 mM betaglycerol phosphate, 10 nM dexamethasone, 10 nM calcitriol and 250 nM ascorbic acid). Medium was then changed twice weekly. The day number 2+X refers to the 2 days of culture in maintenance medium and X days in either osteogenic or maintenance medium. Implants were visualized using an inverted phase contrast microscope (Olympus IX50) or an inverted epifluorescence microscope (Leica), both at x10 magnification. Representative images are shown.

2.5 Raman Spectroscopy of Implants

The implant material was investigated for unwanted chemical reactions and contaminations at various stages using Raman spectroscopy. Raman spectra were obtained using an in-house build Raman microscope. For laser
excitation a Laser Quantum Ventus 532nm Laser (Stockport, UK). The laser is coupled via free space optics into a Olympus BX60 microscope with a 50x objective (Hamburg, Germany) that are fiber-coupled to an Acton SpectraPro 2500i f/6.5 spectrograph, using a 600l/mm grating, blazed at 500 nm and with a Princeton instruments PIXIS 400F 1340×400 pixel CCD camera (Trenton, NJ, USA) operating at −75°C.

For stearic acid 10mW of laser power was applied to the sample and for TCP and implants 30mW was applied. Integration times was 10s with 3 to 10 averages to achieve a comparable SNR. The spectra were offset-corrected and normalized to the maximum value.

2.6 Scanning Electron Microscopy of Implants

Implant microstructure was investigated with scanning electron microscopy (SEM). Samples were prepared for inspection in a SEM (JSM 6480, JEOL), the implant with cells were washed 3 times in distilled water, fixed in 3.7% formaldehyde for 10 minutes at room temperature, dehydrated by air drying, and finally coated with 10 nm of gold in a Cryofox thermal evaporater (Polyteknik A/S, Østervrå, Denmark). The scanning electron microscope (SEM) images were recorded at acceleration voltages of 10 kV and 20 kV and working distance of 15 mm.

2.7 Mechanical Testing of Implant

The mechanical properties of the implants where determined by compression testing in a Zwick Z050 universal testing machine (Zwick Roell, Ulm, Germany). The measured values were: Maximum stress - $\sigma_{\text{max}}$ [N/mm²],
strain at $\sigma_{\text{max}} - dL \%$ and Youngs Modulus – $E$ [MPa]. The test was performed position-controlled with a test of speed 1 mm/s during the entire test sequence.

2.8 Investigating Implant Osteoconductivity with Cell Titer and Alkaline Phosphatase Activity

After 2+7 days of cell culture, the implants were transferred to wells on a 48 well plate to exclude any cells residing on bottom of the culture well from analysis; to the implants were added 40 µL CellTiter and 200 µL Maintenance Medium. After 30 min, 3x70 µL medium was transferred from each well to a black 96 well plate. Viability was measured as fluorescence according to the manufacturer’s protocol using a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany). After determination of viability, the same implants were washed 3 times with PBS, rinsed with TBS, fixed for 30 seconds in 90% Ethanol with 3.7% formaldehyde, the fixative was then removed and the implants were incubated with 250 µL 1 mg/ml 4-nitrophenol phosphate for 20 min. The reaction was then stopped with 150 µL 3 M NaOH. 3x100 µL from each well was then transferred to a clear 96 well plate. Alkaline phosphatase activity was measured as absorbance using a FLUOstar OPTIMA. For each well the average alkaline phosphatase activity was divided by the average viability value to provide a cell number normalized alkaline phosphatase activity measure.

2.9 Investigating Implant Osteoconductivity with Sirius Red and Fast Green Staining

After 2+7 and 2+25 days of cell culture, the implants were transferred to wells on a 48 well plate to exclude any cells residing on bottom of the culture well from analysis. The implants were then washed two times with PBS and were then fixed in Kahle’s fixative (3.7% formaldehyde, 1% acetic acid, 26.88% EtOH) for 10 min at room temperature. The implants were then washed twice with PBS and was then stored at 4°C in 1mL PBS until
staining. For staining a sirius red/fast green staining kit was used (Chondrex, Redmond, WA, USA), the PBS was removed and 300 µL dye solution was added to each well, after 30 min at room temperature the solutions was removed and the wells were washed with 1 mL water until the water was clear (15 times). The wells were photographed and 1 mL of extraction buffer added; after 20-min incubation on a rocking table at room temperature 3 x 200 µL from each well was transferred to a 96-well plate and absorbance was read at 540 nm and 605 nm. The collagen and non-collagenous protein content was then determined using the following equations from the kit manufacturer’s protocol:

\[
\text{Collagen (µg)} = \frac{\text{OD 540 value} - (\text{OD 605 value x 0.291})}{0.0378}
\]

\[
\text{Non-collagenous protein (µg)} = \frac{\text{OD 605 value}}{0.00204}
\]

2.10 Investigating Implant Osteoconductivity with Micro-Computed Tomography Scanning

The same implants were scanned before and after 2+25 days of cell culture. Implants with cells were washed three times in distilled water, fixed in 3.7% formaldehyde for 10 minutes at room temperature, washed three times in distilled water and dehydrated by air drying before being scanned. The implants were wrapped in plastic film and placed in one scanning cylinder that was scanned using a Scanco vivaCT40 using the same settings before and after cell culture. The resulting ISQ files were imported into ImageJ (1.49v) using the KHKs microCT Tools add on [29] and with the following settings: “Downsample by factor 2 in x, y, z (method=average)” and “8-bit-import”. Five slices were then selected at the center of each implant but with a minimum spacing of 10 slices. In each of these slices, the implant was selected as the region of interest, a histogram was generated and the values exported. An identical intensity threshold was then applied to all data
sets to analyze only bone; bone density was then found as: Bone Density = Intensity Level Value(x) * Bone Pixels with Intensity Value (x) /Total Bone Pixels.

2.11 Statistics

Figures 6 and 7 show mean values with sample standard deviation on the error bars. The number of biological replicates is indicated as n in each figure text. Two-tailed t-Tests assuming unequal variance were performed to check for difference between mean values; p < 0.05 was taken to indicate a statistically significant difference.

3. Results

3.1. Ink Testing

At different ratios, all tested ink combinations yielded viscoplastic (shear thinning) suspensions except for the oleyl ester/TCP suspension, which was dilatant (shear thickening). When placed in a syringe and applied pressure, however, all lubricants except oleic acid failed to extrude because the liquid was pressed out first leaving a dry non-extrudable powder in the syringe. Based on this observation, the fatty acids oleic acid, linoleic acid, naphtenic acid, decanoic acid and heated stearic acid were tested and all yielded extrudable TCP formulations. The powder loading achievable depended on the chain length; short chain crotonic acid did not work, whereas the longer oleic, linoleic and stearic acids allowed a higher powder concentration than the medium length naphtenic and decanoic acids. The stearic acid/TCP formulation was chosen for further study as this mixture could be extruded as a viscoplastic melt at 80°C, just above stearic acid’s melting point (69°C). When deposited onto a non-heated stage, the mixture immediately cools and solidifies into a hard material
that supports further layers and overhangs. The implant fabrication technique is summarized in Figure 1, and photographic documentation is available as Supplementary Figure 1.

3.2 Physical Characterization of Implants

Weight loss during sintering was 17.7% (SD: 1.0%, n = 5) corresponding to the pre-sintering weight fraction of stearic acid. Average post-sintering weight was 122.8 mg (SD: 13.6 mg, n = 16); whereas fiber diameter and spacing were 1.6 mm and 0.8 mm, respectively. The calcium phosphate powder was analyzed with SEM, and the individual particles were observed to be 0.5–2 µm in diameter with irregular but rounded shapes (Fig. 2a). Sintered samples (Fig. 2b) contained visible TCP particles, but successful sintering was seen as the particles were fused together (Fig. 2c). Samples cultured for 2+25 days in maintenance or osteogenic medium contained smooth layers of cells and other organic matter that covered the larger part of the TCP surface (Fig. 2d-i). The TCP surface was visible beneath the organic layer at high magnification.

3.3 Mechanical Characterization of Implants

Cubic implants (20 mm x 20 mm x 20 mm) were sintered in two stages (at 400°C for 1 hour and then at 1100°C for 2 hours) or in one stage (directly in a pre-heated oven at 1100°C for 2 hours). Sintering in two stages resulted in the retention of pre-sintering shape, whereas sintering directly at 1100°C resulted in major structural collapse and cracking. Implants from both groups were tested for mechanical properties of compression (Fig. 3). Compressive stress at collapse was 11.6 MPa (SD: 2.1 MPa) for those sintered in two stages and 6.4 MPa (0.9 MPa) for those sintered directly at 1100°C. Young’s modulus was 247 MPa (SD: 108 MPa) for those sintered normally and 104 MPa (SD: 57 MPa) for those sintered directly at 1100°C.
3.4 Chemical Characterization of Implants

To investigate whether contamination or chemical changes take place during implant preparation, the Raman spectra and a photograph of the TCP powder, the stearic acid powder, the non-sintered implants and sintered implants were recorded (Fig. 4). It can be seen that the spectrum of the non-sintered implants is a pure overlay of the TCP and stearic acid spectra, indicating that no visible contamination or chemical reactions take place during the printing. The 400°C sample fluoresced and no Raman spectrum could be obtained; this property is common when biological materials are heated either directly or via photo-induced degradation. The spectrum of the fully sintered implants is identical to that of the TCP powder, which indicates the stearic acid and any other organic matter is completely removed after sintering. All samples were white except the sample that had been heated to 400°C for 1 hour with no further sintering; the brown/gray color of this sample is likely due to stearic acid carbonization taking place at 400°C. Any developed carbon subsequently burns away during the 2-hour step at 1100°C, as indicated by the white color and pure TCP Raman spectrum of the fully sintered implant.

3.5 Cell Adhesion and Proliferation on Implants

To investigate whether the printed implants supported cell adhesion and growth, hMSCs (eGFP⁺) were seeded onto implants and visualized at 24h and 48h using an inverted epifluorescence microscopy. The cells were observed to adhere and spread out on the implant surface. In a different experiment, hMSCs (eGFP⁺) were seeded onto implants, and after 2 days in maintenance medium, osteogenic medium was added to half the samples. On day 2+7, the implants were visualized with an inverted microscope (Fig. 5). Although it was
impossible to view the non-fluorescent cells on the ceramic structure itself, a thick cell layer was observed within the pores that adhered to the ceramic walls; no significant differences between the groups were observed. On day 2+25 this layer had grown and in some cases covered all the pores (data not shown).

3.6 Osteoconductivity of Implants

To study osteoconductivity, hMSCs (eGFP) were seeded onto implants and were, after 2 days in maintenance medium, cultured in either maintenance medium or osteogenic medium. Cell titer and alkaline phosphatase activity were measured spectrophotometrically in the same samples on day 2+7. Change in mineralization for a different sample set was measured by µCT on day 2+25 (Fig. 6); this sample set was the one also investigated by SEM (Fig. 2). The medium did not affect the cell titer \( (p = 0.11) \). Alkaline phosphatase activity, non-normalized \( (p = 0.00044) \) and normalized to cell titer \( (0.0022) \), was affected by the medium, increasing 3.9 and 4.1 times maintenance medium and osteogenic medium, respectively. The medium also affected the degree of mineralization \( (p = 0.037) \), which decreased 1.9% in and increased 3.0% in osteogenic medium.

Implants were stained for protein and collagen deposition on day 2+7 and day 2+25 using a combined Sirius red and fast green assay (Fig. 7). In the osteogenic medium samples, collagen was deposited over the entire implant. The stains were extracted and quantified using spectrophotometry. On day 2+7, there were no differences in overall protein deposition \( (p = 0.47) \), but the osteogenic medium led to 67% higher collagen deposition \( (p = 0.0015) \). On day 2+25, collagen deposition was identical in the two groups \( (p = 0.28) \), but protein deposition was 60% higher in maintenance medium \( (p = 6.6 \times 10^{-6}) \). Collagen deposition increased between day 2+7 and day 2+25 in the maintenance medium group \( (p = 7.6 \times 10^{-7}) \) but not in the osteogenic medium group \( (p = 0.81) \). Protein deposition increased between day 2+7 and day 2+25 in the maintenance medium group \( (p = 2.8 \times 10^{-6}) \) and in the osteogenic medium group \( (p = 0.00011) \).
4. Discussion

The use of fatty acids to bind TCP mimics nature’s own approach. Acidic SIBLING proteins bind and coordinate calcium and by doing so are able to nucleate and bind hydroxyapatite [30]. This interaction is facilitated by ionic bonding between the calcium and carboxylate side chains of acidic amino acids. The same bonding likely takes place when fatty acids are combined with TCP and facilitates bonding even when pressure is applied. This is in contrast to all of the non-ionic lubricants that were tested. The aliphatic groups then likely result in improved lubrication through lowered shear stress and inter-particle attraction that enable the extrusion of inks with a high powder fraction. The fatty acid/TCP mixtures could be extruded after storage and reheating, and when deposited onto a stage, they solidified immediately and became strong enough to support the layers above. In addition, these mixtures did not require dehydration before sintering. Combined these features significantly reduces the overall fabrication time and complexity compared to aqueous formulations.

Implants with heights of 2 cm could be sintered without sagging despite stearic acid/TCP being viscoplastic above stearic acid’s melting point. Fatty acids are known to carbonize when heated [31], and this probably happens during the 1-hour 400°C pre-sintering step because the color of the sample changed from white to brown after this step. The resulting carbon may stabilize the structure, and when the temperature is raised further, the sintering of the TCP particles likely proceeds to replace the compressive strength lost as the carbon burns away. This theory is supported by the fact that sagging occurred in samples sintered directly at 1100°C, where combustion was rapid and the organic matter probably burned away before sintering occurred. Regardless of the mechanism, implant sintering was possible without sagging and resulted in chemically pure TCP that matches previous published spectra of TCP [32].
The implants contained macropores with a diameter of 800 µm (Fig. 2). Osteogenesis seems to be optimal when macropore diameter is greater than 400 µm [33-34], and one recent study found that pores of 1000 µm were optimal [35]. In the compressive test, the implants had an average Young’s modulus of 247 MPa and compressive strength of 11.6 MPa, both values lie within the range of normal human skull bone, Young’s modulus and compressive strength ranging from 72 MPa to 3654 MPa (average: 1393MPa) and from 5.3 MPa to 108.2 MPa (average: 36.5MPa), respectively [36]. The values are comparable with those achieved with aqueous TCP formulations [37]. A high activity of alkaline phosphatase on day 2+7 in cell culture was observed, alkaline phosphatase releases phosphate for calcium phosphate mineralization, and an increase in mineralization was observed on day 2+25 using µCT. This likely increases the strength further, especially as it is known that small increases in bone mineral density result in major increases in mechanical strength [38]. The tensile, flexural or shear strengths of the implants were not measured. These strengths are the mechanical stress modes wherein calcium phosphate and most other ceramics fail. In bone, these strengths are provided by collagen, and it is known that polymer infiltration of TCP may improve tensile strength [39]. It is therefore probable that the rapid collagen deposition improves the tensile strength. The compressive strength of the sintered implants is within the range of that achieved in other studies on 3D printed TCP implants such as 10.95 MPa [40], 5.74 MPa [41] and 12.01 MPa [42], and it is likely sufficient for using them in non-load bearing situations such as craniofacial bone replacement. If cultured for 1 or more weeks prior to implantation, the deposition of collagen on and additional mineralization of the implants may increase their strengths sufficiently for load bearing applications. It is also likely that a higher sintering temperature would increase the mechanical strength, although this might increase the duration of the sintering process.

MSCs were seeded for generating the bone in vitro; however, this may not be necessary in vivo because MSCs have been found to migrate from bone marrow to calcium phosphate implants where they stimulate bone formation even if these implants are placed ectopically [43]. In osteogenic medium, bone development took
place with the established series of events: initial collagen deposition and alkaline phosphatase secretion followed by mineralization, demonstrating that the implants are osteoconductive. In maintenance medium, a low level of alkaline phosphatase activity and collagen deposition was also observed, but mineralization did not increase.

With pre-prepared inks, 8 cm² of implant could be 3D printed in less than an hour; with additional 4–5 hours of sintering, implant preparation was completed well within 1 workday. This was accomplished using a low-cost 3D printer and sintering oven. In the future, the short, simple and inexpensive workflow enabled by the fabrication technique detailed here may therefore allow hospital departments to 3D print their own custom implants in-house for both non-elective and elective operations. This is a distinct advantage over current setups where an external partner fabricates custom implants off-site. Such setups are expensive, typically incur longer production times and may be subject to logistical delays. In-hospital 3D printing could still be carried out with the assistance of an external and off-site CAD expert that receives digital patient scanning data and returns a finished CAD model, after which this CAD model could be printed locally at the hospital.

While the present study focused on TCP implants, we found that other powders could also be 3D printed by the technique described here. Metallic copper powder and ceramic copper oxide powder were both printed with success (Supplementary Figure 2). This potentially extends the method to include fast and inexpensive 3D printing of metals and other ceramics for medical and non-medical applications.

5. Conclusion

In conclusion, porous TCP implants may be additively manufactured using robocasting of fatty acid/TCP inks. The inks are storable and reusable, and the total fabrication time including ink preparation, printing and
sintering is less than 5 hours for 8 cm² of implant. The implants retained their pre-sintering shape after sintering, they were chemically unchanged by the printing and sintering process, and they possessed clinically relevant mechanical strength. In vitro, mesenchymal stem cells were able to grow on the implants, secrete collagen and alkaline phosphate and mineralize the implant. Additional studies are required that include implants with larger and more complex geometries. Given the results obtained, however, we believe that the ink described here may be used as a key component in future rapid and simple manufacturing methods that enable osteoconductive, resorbable and patient-specific implants to be made locally at hospitals.

6. Acknowledgements

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7. Author Contributions

CS, MBJ and MØA devised the 3D printing technique, CS and MBJ fabricated the implants used, ND conducted the CT scan, MABH carried out the Raman analysis, OA obtained the SEM pictures, SWB helped CS and MBJ with the mechanical testing, TT and MK provided clinical guidance for the work, MØA carried out the cell culture and analysis, supervised CS and MBJ, wrote the draft manuscript and coordinated the study. All authors contributed to the final paper.

8. Financial Disclosure
MØA, CS and MBJ are named as inventors on a patent covering the printing technology described in the paper.

At the time of submission, the patent was owned by the University of Southern Denmark.

9. Figures

Figure 1. A schematic representation of how the ink and printing concept probably works. Fatty acids bind the calcium phosphate particles by electrostatic interaction between the carboxylic acid groups and exposed calcium atoms. The hydrophobic tails project away from the particles and facilitate inter-particle lubrication, allowing the fluidization of the ink even at high particle loading. The ink is heated and deposited on a build surface where it solidifies quickly as it cools.
Figure 2. Scanning electron microscopy images. The following samples were imaged: TCP powder prior to use (a), sintered TCP implants at x25 and x1000 magnification (b and c), cell-seeded TCP implants after 2+25 days of cell culture in maintenance medium at x25 magnification (d), cell-seeded TCP implants after 2+25 days of cell culture in osteogenic medium at x30 and x500 magnification (e and f).
Figure 3. Compression testing of cubic (8 cm$^3$) TCP implants. Strain-Stress curves for compression testing were recorded (top figure) for two-stage sintered (400°C and 1100°C, $n=3$) and one-stage sintered implants (1100 °C, $n=4$), displayed are the average stress values of the implants from each group at the given deformation. Representative photographs of the sintered implants as seen from their top and side were taken before compression testing (bottom left and right figure, respectively), the two-stage sintered implants are seen to the left of each image.
Figure 4. Characterization of the sintering process. Raman spectra were collected (left figure) of sintered implants (top), non-sintered implants (upper-middle), TCP powder (lower-middle), stearic acid powder (bottom). A photograph was taken (right figure) of TCP powder (left-upper), stearic acid (left-lower), non-sintered implants (center-left), implants heated to 400°C for 1 hour (center-right) and fully sintered implants (right).
Figure 5. Inverted microscopy pictures of cells growing in the implant pores on days 1, 2 and 2+7.
Figure 6. Cell titer and alkaline phosphatase activity (ALP) on day 2+7 (n=4) and change in mineralization on day 2+25 as compared to day 0 (n=4). Cell Titer and alkaline phosphatase activity was measured on the same samples.
Figure 7. Staining of collagen (red) and protein (green) on the same implants \((n=4)\) on day 2+7 (top). After staining on days 2+7 and 2+25 the stains were extracted from the implants \((n=4)\) and quantified by absorbance spectrophotometry (right) \((n=4)\). MM and OM indicates maintenance medium and osteogenic medium.

10. References


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